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TO OUR READERS!

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We have the honour to inform you of the change in title of our journal.

As of 1976, the former "Acta Alimentaria Academiae Scientiarum Hungaricae" will be published under the title "ACTA ALIMENTARIA".

This change will not affect the present scope, volume or editorial policy of the journal in any way.

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THE EDITORIAL BOARD

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THIN-LAYER CHROMATOGRAPHIC INVESTIGATION OF VOLATILE NEUTRAL CARBONYL COMPOUNDS FORMED DURING COOKING OF PORK

E. KOZMA-KOVÁCS

(Received March 21, 1974)

The carbonyl compounds were obtained by an appropriate extraction procedure from defatted and twice minced *semimembranosus* muscle of pig cooked in the laboratory and were investigated in the form of 2,4-dinitrophenylhydrazine-(DNPH)-derivatives.

Using the data published in the literature a multi-step thin-layer chromatographic method was developed for the determination of the DNPH-derivatives, *i.e.* for the qualitative assessment of the neutral carbonyl compounds formed during boiling of the meat.

The method provided evidence on the presence of mono- and dicarbonyl compounds in cooked meat.

The following carbonyl compounds were identified:

– of the *aliphatic monocarbonyl* compounds: formaldehyde, acetaldehyde, propionic aldehyde, iso-butyric aldehyde, butyric aldehyde, iso-valeric aldehyde, valeric aldehyde, caproic aldehyde, oenanthic aldehyde (oenanthal) and acetone;

– of the *aliphatic alpha-dicarbonyl* compounds: glyoxal and diacetyl.

Six saturated and one unsaturated aldehyde, three ketones and, of the bis-derivatives, two alpha-diketones could not yet be identified.

A number of researchers have recently studied the chemical structure of the volatiles of meat aroma. The majority of the pertaining literature refers to the separation and identification of groups of compounds formed in various types of meat upon cooking. Thus HIRAI and co-workers (1968, 1973) studied the hydrocarbons, alcohols, esters, carbonyl compounds, sulphides and aromatic heterocyclic compounds which all contribute to the flavour of boiled beef.

PERSSON and VON SYDOW (1973) performed similar experiments with canned beef, while WILSON and KATZ (1972) studied the flavour of boiled chicken according to the groups of compounds present.

Of the volatiles responsible for the flavour of cooked beef, many researchers investigated only a single group of compounds, *e.g.* the carbonyl compounds, as important flavour constituents. PIPPEN and co-workers (1958), MINOR and co-workers (1965a), PIPPEN and NONAKA (1960), studied the carbonyl compounds in boiled chicken, SANDERSON and co-workers (1966) the same in boiled beef and HALVARSON (1973) in sausages.

The aim of the present work was to determine the volatile neutral carbonyl compounds in boiled pork.

In their studies on the flavour of boiled pork and beef HORNSTEIN and CROWE (1960) found no substantial difference in the identified carbonyls of the two meats. The presence of normal alkanals of 2 to 11 carbon atoms, of acetone and of certain 2-enals and 2,4-dienals could be proved.

MERRITT (1972) investigated the carbonyl compounds and other flavour components in various irradiated meats, including pork. This author claims that of the carbonyl compounds in boiled pork only caproic aldehyde and acetone are present in substantial quantities.

The number of papers dealing with the carbonyl compounds of boiled pork is relatively small hence we studied all available literature on the flavour of boiled pork, pork products (ham, sausage) and of other boiled meat (chicken, lamb, beef) which included a detailed discussion of the carbonyl compounds as flavour constituents or was restricted to the study of these compounds only.

The compounds identified by us and the literature data are summed up in Table 1. The results will be discussed in the paragraph on our conclusions.

In meat subjected to heat treatment carbonyl compounds might be formed by complex reactions from amino acids, sugars, lipids and other non-volatile compounds. Several aldehydes and ketones might be the decomposition products of saturated fatty acids (DAY & LILLARD, 1960; SCHWARTZ *et al.*, 1963).

According to our findings a great variety of carbonyl compounds is formed in the course of these reactions though their quantities are rather small. The volatile carbonyls were separated in the form of their DNPH-derivatives from the other flavour components.

For the thin-layer chromatographic tests the methods of BADINGS and WASSINK (1963), BEYER and KARGL (1972), RONKAINEN (1967), SCHWARTZ and co-workers (1968) and of WALTHER (1967) were used after appropriate modification.

1. Materials and methods

1.1. Separation of the volatile carbonyl compounds from boiled pork

Defatted and twice minced *semimembranosus* muscle of pig was used in the experiments.

The volatile carbonyl compounds were extracted from the boiled meat by means of NONAKA's method (1971) in the following manner.

The minced meat–water (1 : 2) homogenate was poured into a three-neck flask provided with stirrer, condenser and a ground-glass joint for the introduction of a nitrogen stream. The suspension was boiled over a Bunsen-flame under constant stirring for about 3 hours. The volatile flavour constituents were led through the condenser into a trap containing 50 ml of DNPH-

reagent (the solution of 0.24 % of 2,4-dinitro-phenylhydrazine in 2 *N* HCl) by bubbling through the nitrogen at a constant rate of 30–40 ml per min. The DNPH-reagent in the traps (gas washing flasks) bound the carbonyl compounds. At the end of boiling a precipitate appeared in the first trap, while in the second trap, inserted for checking, there was no precipitate.

Since heat treatment results in the formation of small amounts of a great variety of carbonyl compounds, it is essential to provide for the complete extraction of the flavour.

For this reason the carbonyl compounds which remained in the broth of the boiled meat were removed by way of the above distillation method, but without stirring.

After having established that the carbonyl compounds separated in this manner were the same as those obtained from the boiled meat, the same trap was used for their binding.

1.2. Preparation of the DNPH-derivatives of carbonyl compounds for thin-layer chromatographic tests

The precipitate collected in the trap was allowed to stand overnight at room temperature and was filtered next day through a sintered glass filter G4. The filtrate was boiled for 20 min to precipitate the DNPH-derivatives of the di- and oxo-carbonyls. After cooling to room temperature the precipitate was removed by filtration through the same filter, washed with 2 *N* HCl till the filtrate became colourless, washed next with distilled water to remove the acid, dried and dissolved in carbonyl-free ethyl acetate.

Carbonyl impurities had to be removed from all the solvents used in the tests (VOGEL, 1954; SCHWARTZ & PARKS, 1961; ERDEY, 1956; PELEG & MANNHEIM, 1970).

1.3. Survey of the thin-layer chromatographic testing of "all" carbonyl DNPH-derivatives

The multi-step thin-layer chromatographic method used for the separation of the DNPH-derivatives of neutral carbonyls is shown in the form of a flow-sheet in Fig. 1.

The solution in ethyl acetate of the carbonyl compounds obtained from boiled meat and prepared according to para. 1.2 contains the DNPH-derivatives of "all" the carbonyl compounds (aliphatic, aromatic and di-carbonyls). In the first step of the chromatographic process derivatives of the same groups of compounds were separated (para. 1.3), followed by multi-step chromatography of each carbonyl group.

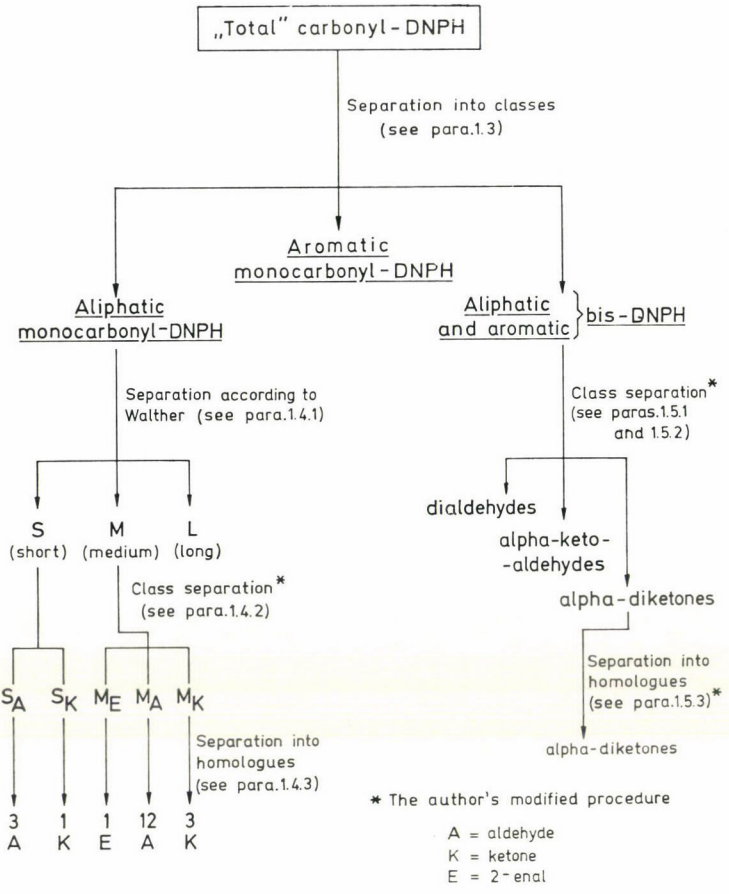

Fig. 1. Separation of the DNPH-derivatives of neutral carbonyls by means of multi-step thin-layer chromatography

After the preliminary separation of the aliphatic monocarbonyls according to chain length (para. 1.4.1), the aldehyde, ketone and 2-enal fractions (no 2,4-dienals were detected) were separated (para. 1.4.2), finally the homologues were identified by means of model substances (para. 1.4.3).

No aromatic carbonyl compounds were found in boiled pork.

In thin-layer chromatography of the dicarbonyl compounds first alpha-diketones were separated (para. 1.5.1) and at the same time the presence of alpha-keto-aldehydes and -di-aldehydes was established. Next, the various components were identified (para. 1.5.3).

In the following paragraphs a detailed account will be given of the various steps in the chromatographic procedure.

In the first step of thin-layer chromatography, separation of the DNPH-derivatives of "all" the carbonyls into groups of compounds was performed

according to BEYER and KARGL (1972) on a $ZnCO_3$ adsorbent in a 99.5 : 0.5 mixture of pyridine and water. By means of this method the carbonyl derivatives can be divided on the basis of their original structure — according to the R_f values and difference in colour — into three groups: aliphatic mono- as well as aromatic mono- and dicarbonyls.

The aliphatic monocarbonyls were found near the front-line, their colour was yellowish brown. Below them appeared the pinkish purple aromatic monocarbonyl DNPH-derivatives, while the derivatives of both the aliphatic and aromatic dicarbonyls were situated in the form of red, blue and violet spots and stripes, near the start.

Using a $ZnCO_3$ layer of preparative thickness (0.50 to 0.75 mm thick) the various groups of carbonyl compounds could be obtained in larger quantities. The stripes were removed from the plate and the compounds extracted from the adsorbent by means of ethyl acetate.

Since the DNPH-derivatives of carbonyl compounds obtained from boiled pork represented only two groups of compounds, namely aliphatic monocarbonyls and various dicarbonyl derivatives, the separation of these two groups shall be discussed only.

1.4. Monocarbonyl DNPH-derivatives eluted from the $ZnCO_3$ layer

In order to be able to identify the separated components by means of model substances the DNPH-derivatives of the mono-carbonyls dissolved in ethyl acetate have to be separated in three subsequent chromatographic steps.

The monocarbonyl fractions are always eluted after collection from several layers. Ethyl acetate free of carbonyl impurities is used for elution. Prior to further chromatographing the eluates are concentrated by evaporation to 2 or 3 ml.

On the chromatogram the separated carbonyl DNPH-compounds appear in a yellow colour without any treatment. The shades obtained after alkaline treatment are characteristic of the various groups of compounds and depend on both the reagent used and the nature of the adsorbent.

The shades observed after treatment with the reagent will be discussed in detail when describing the later steps in chromatography which involve classification of the substances on the basis of the colours of the spots.

The layers were either sprayed with ethanol containing 12% of NaOH, or placed in a closed glass tank filled with diethylamine vapours. Both treatments resulted in the same colours. Sodium hydroxide treatment led to the formation of more stable colours.

Diethylamine is a reversible colour reagent and evaporates easily from the adsorbent as soon as the layer is lifted from the closed tank containing the diethylamine vapours.

1.4.1. Preliminary separation of the DNPH-derivatives according to chain length. By means of this step in chromatography it is possible to separate the monocarbonyl derivatives into compounds with short (*S*), medium (*M*) and long (*L*) carbon chains.

WALTHER's method (1967) employed in this step consists in applying, beside the DNPH-derivatives of monocarbonyls, acetone and C_{10}-aldehyde-DNPH to the Kieselgel adsorbent and in running the layer four times in a mixture of benzene and hexane.

After drying and development the chromatogram is divided into three parts depending upon the positions of the spots of acetone and C_{10}-aldehyde-DNPH, resp.

The spots of the carbonyl derivatives with short carbon chain (*S*) will appear between the start and the acetone spot, those with carbon chains of medium length (*M*) between the spots of acetone and of the C_{10}-aldehyde-DNPH, while the spots of carbonyl derivatives with long carbon chains (*L*) will be located between the spot of C_{10}-aldehyde-DNPH and the front line.

Chromatography aims at preventing overlapping between members with close carbon atom numbers of the monocarbonyl derivatives comprising several homologous series (aldehydes, ketones, 2-enals and 2,4-dienals) in subsequent chromatography in which the separation of the monocarbonyl fractions into classes is attempted.

1.4.2. Separation of monocarbonyl-DNPH derivatives previously divided into classes according to chain-length. According to SCHWARTZ and co-workers (1968) the monocarbonyl derivatives can be divided into four fractions (aldehydes, ketones, 2-enals and 2,4-dienals) on the basis of the nature of their functional group and the saturation of their carbon chain.

Instead of the layer recommended by the authors (consisting of MgO and analytical grade Celite) we used an adsorbent made up of MgO, Celite and Kieselgel G and changed the composition of the solution used in the runs. The MgO and solvents employed in these tests were of analytical grade.

For the preparation of the layer 20 g of Kieselgel G, 6 g of Celite 545, chromatographic grade, and 3 g of MgO (after having been screened with the Celite through a 100 mesh sieve) were suspended in 75 ml of methanol containing 1% of KOH and five 20 cm $\times$20 cm plates of 0.30 mm thickness were prepared. The plates were dried at room temperature and used after 30 min activation.

The eluates (*S*, *M* and *L*) of the zones, separated according to the chain-length of the carbonyl compounds, were applied as separate bands to the plates. The layers were run, instead of the hexane – chloroform (95 : 25) solvent mixture recommended by the authors, in an 80 : 14 : 6 mixture of hexane – benzene – ethyl acetate. Chromatographing was repeated (duration: about 2 $\times$30 min).

the layer. The layers were run in a 95 : 5 chloroform – methanol mixture for about 80 min. The blue and violet spots began to appear during chromatographing (due to the alkaline nature of the adsorbent).

The bis-DNPH derivatives of the dicarbonyl compounds of meat flavour were separated into three aliphatic dicarbonyl groups on the basis of the R_f values of the model compounds. The sequence of dicarbonyls on the layer was as follows:

– *dialdehydes*, in the field between the bis-osazones of glyoxal and methylglyoxal;

– *alpha-keto-aldehydes*, in the band between the bis-osazones of methylglyoxal and diacetyl;

– *alpha-diketones*, in the field between the spot of diacetyl-bis-osazone and the front line (diacetyl-osazone is adsorbed near the front-line).

The bis-carbonyl derivatives could be eluted from the magnesium oxide adsorbent only after wetting by 2 *N* HCl and subsequent extraction of the bis-osazones from the aqueous suspension by means of pyridine. The pyridine solution was filtered, evaporated to dryness in vacuum and the residue taken up in ethyl acetate.

1.5.2. Investigation of the dicarbonyl-osazones in the flavour of cooked meat by means of RONKAINEN's (1967) *method.* Instead of the paper Whatman No. 3 recommended by the author we employed Polygram Sil G precoated plates. The solvent system used in the runs was the same as the one described above. The plates were activated prior to use at 105 °C for 30 min.

The dicarbonyl-osazone groups appear in the same order on the chromatogram, as in the method described in para. 1.5.1. Due to the regular point-like separation of the components this chromatogram can be used for the identification of the components.

1.5.3. Separation of the bis-osazones of aliphatic alpha-diketones. SCHWARTZ and co-workers (1968) succeeded in separating the alpha-diketones and alpha-keto-aldehyde homologues on a Microcel T-38 layer impregnated with Carbowax 400.

Using Kieselgur G adsorbent impregnated with Carbowax 400 we were able to separate, with the solvent recommended in the literature, only the components of alpha-diketones.

The layer was prepared and activated under the conditions described in para. 1.4.3.

With the available model alpha-diketones it was possible to identify the compounds by means of this method.

2. Results

2.1. Quantity of volatile carbonyl compounds formed during cooking of pork

The "total" amount of carbonyl-DNPH-derivatives obtained as the average in several series of experiments in the course of boiling of 1 kg of *semimembranosus* muscle was 46 mg. About 93 % of this amount were aliphatic monocarbonyl derivatives and 7 % a mixture of dicarbonyl-bis-DNPH-derivatves.

2.2. Separation of the "total" carbonyl-DNPH-derivatives into groups of compounds

When the ethyl acetate solution of the carbonyl derivatives of boiled meat (prepared according to para. 1.2) was chromatographed on a zinc carbonate layer (along with the model substances, in a 99.5 : 0.5 pyridine – water mixture) the chromatogram shown in Fig. 2 was obtained.

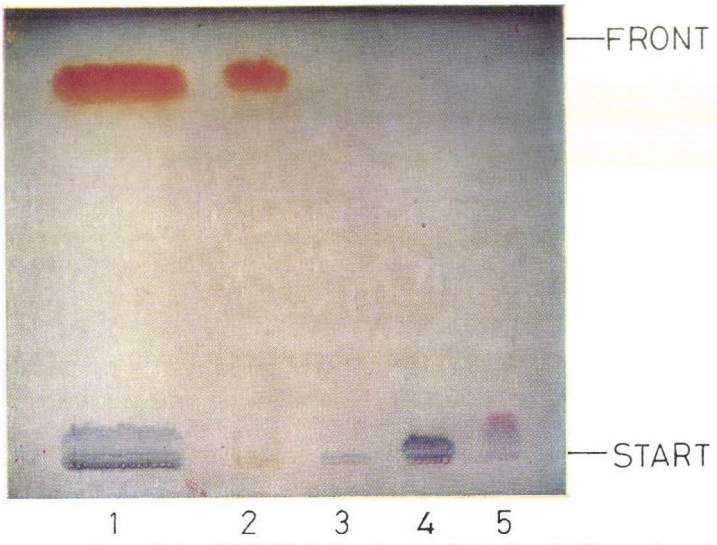

Fig. 2. Group separation of the DNPH-derivatives of total volatile carbonyls formed during cooking of pork. Layer: zinc carbonate. Solvent: pyridine – distilled water (99.5 : 0.5). Samples: 1. DNPH-derivatives of the carbonyl compounds in pork flavour (0.06 ml); 2. aliphatic monocarbonyl standard: acetone-DNPH (0.02 ml); 3. glyoxal-bis-DNPH standard (0.02 ml); 4. methylglyoxal-bis-DNPH standard (0.02 ml); 5. diacetyl-bis-DNPH standard (0.02 ml)

It appears clearly from the chromatogram that when pork is boiled mainly volatile mono- and dicarbonyls are formed. The yellowish brown spots of the aliphatic monocarbonyls (with the highest R_f values) are located below the front-line. Near the start line (with the lowest R_f values) the blue, violet

The order of adsorption and colour of the monocarbonyl groups separated on the layer were from the front line to the start, in agreement with the above cited authors, as follows:

alkanone (saturated ketones, *K*) yellowish brown
alkanal (saturated aldehydes, *A*) reddish brown
2-enal (aldehydes or ketones with olefin bond, *E*) pink
2,4-dienal (aldehydes or ketones with conjugated bond, *D*) red.

The method has the drawback that it is difficult to distinguish the characteristic colours of the different classes, particularly if the concentrations of the groups are very different. This drawback will be manifest to some degree in the next step, when the homologues are separated according to the carbon atom number, nevertheless, this step, *i.e.* separation according to the class of compounds, cannot be omitted from the sequence of chromatographic steps.

1.4.3. Division of the DNPH-derivatives of monocarbonyl homologues into their components. For the division of the monocarbonyl homologues according to the number of carbon atoms in the chain usually impregnated layers are employed (BADINGS & WASSINK, 1963; URBACH, 1963). We adopted WALTHER's method (1967).

The principle of the method is as follows. The monocarbonyl-DNPH homologues of the various classes can be separated by thin-layer chromatography on a Kieselgur G layer impregnated with polyethylene glycol and run twice in cyclohexane.

After alkaline spraying spots appeared on the layer and the colour of the spots was the same as that found on the layer after separation of the classes of compounds, though in this step the various groups of compounds were easier to distinguish. The two steps of the chromatographic procedure complement each other.

This final step involves the identification of the members of the separated homologue series, provided we have appropriate model substances at our disposal.

1.5. Further investigation of the bis-DNPH-derivatives obtained by elution of the $ZnCO_3$-layer

In the eluate containing the bis-osazones various dicarbonyl derivatives might also be present (para. 1.3). SCHWARTZ and co-workers (1968) succeeded in separating from the aliphatic alpha-dicarbonyl mixtures glyoxal, an alpha-keto-aldehyde and an alpha-diketone fraction in a single chromatographic step. We used a modified version of their method and found that it is possible to separate the dicarbonyl derivatives also from the bis-osazones of meat flavour.

The bis-DNPH-derivatives of the first three homologues of alpha-dicarbonyls, namely of glyoxal, methylglyoxal and diacetyl, were studied by RONKAINEN (1967) by way of paper chromatography. We adapted his method to thin-layer chromatography and tried it for the dicarbonyl-osazones of meat flavour. The results were in good agreement with those obtained by the adapted chromatographic method of SCHWARTZ and co-workers (1968).

The above three aliphatic dicarbonyl derivatives, as well as the alpha-diketone components were separated, the latter by means of the modified method of SCHWARTZ and co-workers (1968).

The same principles are valid for the chromatographic separation of bis-osazones as for that of the monocarbonyl derivatives.

The bis-derivatives of the alpha-diketones were collected from several layers and then eluted. The layers were developed either by spraying with alkaline ethanol or by means of diethylamine vapour when blue and violet spots appeared.

It has to be mentioned that, depending upon whether one or two molecules of 2,4-dinitrophenylhydrazine participated in the formation of the DNPH-derivative, the model diacetyl compound appeared on the chromatogram as a purple or bluish violet spot. The model diacetyl-DNPH prepared by us contained both derivatives. The above finding is supported by literature data (REICH & HEFLE, 1956).

1.5.1. Separation of the bis-osazones of aliphatic alpha-dicarbonyls. As already mentioned, by means of the method of SCHWARTZ and co-workers (1968) it is possible to separate the aliphatic dialdehyde, alpha-keto-aldehyde and alpha-diketone-bis-DNPH-fractions from the various dicarbonyl derivatives of meat flavour.

Instead of the MgO plus analytical grade Celite described in the literature we used analytical grade MgO as adsorbent. The solution used in the runs gave good results without modification.

The principle of the method is as follows. Beside the DNPH-derivatives of the bis-carbonyl compounds of meat flavour (the first members of the series of aliphatic alpha-dicarbonyl homologues) glyoxal-, methylglyoxal- and diacetyl-bis-osazone were also applied to the magnesium oxide adsorbent. The layer was developed in a chloroform–methanol solution.

The layer was prepared in the following manner: 17 g of magnesium oxide, analytical grade, were passed through a 100 mesh sieve and suspended in 65 ml of distilled water. From this suspension 0.25 mm thick, 20 cm $\times$20 cm layers were prepared which were air-dried and then activated at 100 °C for one hour. The layers must be used on the day of preparation.

Method. The ethyl acetate solution of the bis-DNPH-osazones of the dicarbonyl compounds from meat flavour, as well as the solutions of glyoxal-, methylglyoxal- and diacetyl-bis-osazone were applied in the form of spots to

and purple spots of the dicarbonyl derivatives are to be found (the mono-DNPH of diacetyl giving the purple spot). The DNPH-derivatives of aromatic carbonyls are missing: there are no spots in the central zone of the chromatogram.

Hence there are mono- and dicarbonyl derivatives in the flavour substances of boiled pork. In addition, the chromatogram permits of conclusions

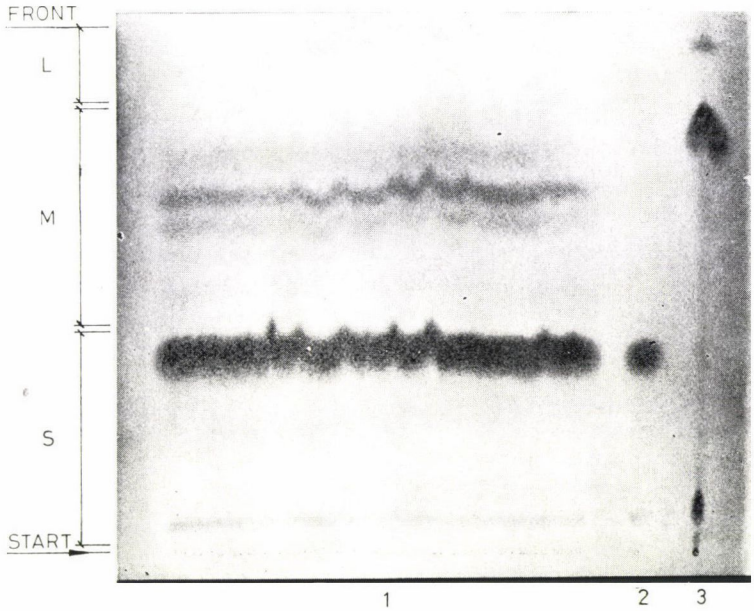

Fig. 3. Preliminary separation according to chain length of the monocarbonyl-DNPH fraction of volatiles formed during cooking of pork. Layer: Kieselgel G. Solvent: benzene – hexane (60 : 40). Samples: 1. monocarbonyl-DNPH-derivative of pork flavour (0.05 ml); 2. acetone-DNPH standard (0.02 ml); 3. C_{10}-aldehyde-DNPH standard (0.02 ml); L = long chain, M = medium chain, S = short carbon chain monocarbonyl derivatives

regarding the presence of glyoxal, methylglyoxal and diacetyl. The R_f value of the monocarbonyls is around 0.9, that of the dicarbonyls between 0.02 and 0.1.

The two groups of carbonyl compounds were eluted from the layer and subjected to further chromatographing.

2.3. Thin-layer chromatography of aliphatic monocarbonyl-DPNH-derivatives eluted from the zinc carbonate layer

2.3.1. Preliminary separation of the monocarbonyl fraction according to chain length. Chromatographing according to WALTHER (1967) resulted in the separation shown in Fig. 3.

It appears from Fig. 3 that the monocarbonyl-DNPH-derivatives in boiled pork contain large amounts of compounds with short carbon chains (S), fair amounts of compounds with medium sized carbon chains (M) and only very little of carbonyl compounds with long chains (L). For this reason

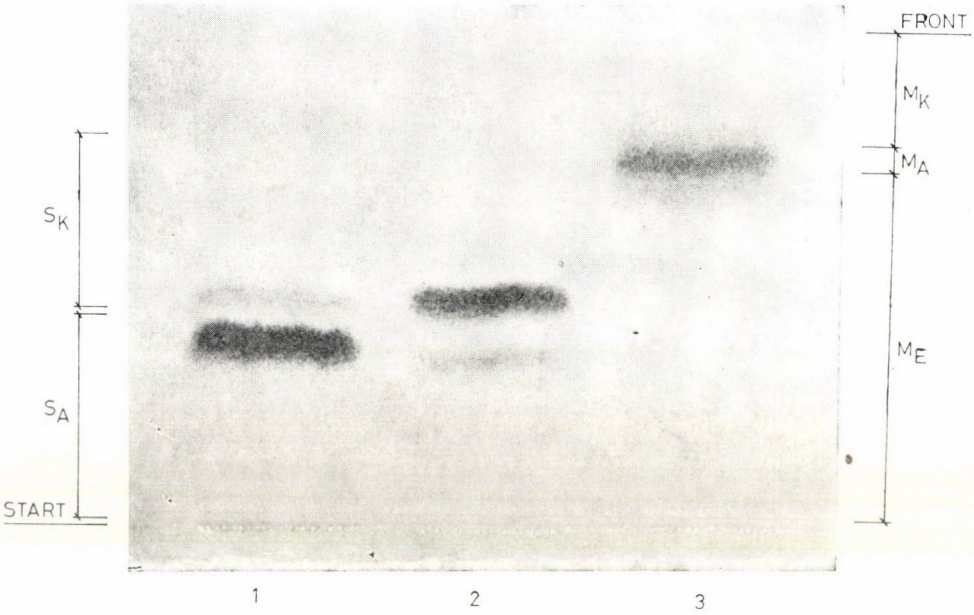

Fig. 4. Class separation of the monocarbonyl-DNPH-derivatives of different (S, M) chain lengths from the volatiles formed during cooking of pork. Layer: Kieselgel G–Celite 545–MgO (20 : 6 : 3). Solvent: hexane – benzene – ethyl acetate (80 : 14 : 6). Samples: 1. Short chain (S) fraction of the monocarbonyl-DNPH-derivatives from pork flavour (0.08 ml); 2. mixture of acetone-DNPH and acetaldehyde-DNPH standards (0.06 ml); 3. medium-chain (M) fraction of the monocarbonyl-DNPH-derivatives from pork flavour (0.06 ml)

only compounds with short and medium length carbon chains were subjected to further investigation.

2.3.2. Separation of the classes of compounds in the zones eluted according to chain length. The result of the separation performed according to the method of SCHWARTZ and co-workers (1968) is shown in Fig. 4.

The chromatogram indicates the separation of the compounds with short carbon chains (S) into aldehyde (S_A) and ketone (S_K) fractions. The zones of the compounds with medium length chains (M) contained 2-enals (M_E), aldehydes (M_A) and ketones (M_K).

2.3.3. Separation according to chain length of the homologues obtained after the classification of the monocarbonyls. The results obtained by the procedure described in para. 1.4.3 are illustrated in Fig. 5.

By means of this method and model substances the following compounds could be identified: formaldehyde, acetaldehyde, propionaldehyde, isobutyraldehyde, butyraldehyde, iso-valeraldehyde, valeraldehyde, caproaldehyde, oenanthal, acetone, 2-enal compounds and high molecular weight aldehydes and methyl ketones.

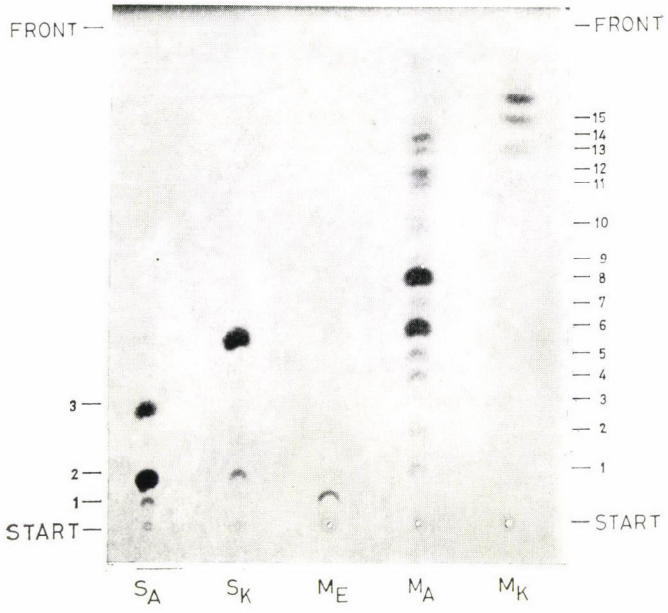

Fig. 5. Separation of the monocarbonyl-DNPH-derivatives from volatiles formed during cooking of pork according to carbon atom number. Layer: Kieselgur G impregnated with polyethylene glycol 400. Solvent: cyclohexane (repeated run). Samples: 1. Group S_A of the short-chain (S) fraction of pork flavour; 2. Group S_K of the short-chain fraction of pork flavour; 3. Group M_E of the medium-chain fraction of pork flavour; 4. Group M_A of the medium-chain fraction of pork flavour; 5. Group M_K of the medium-chain fraction of pork flavour (a sample of 0.05 ml was taken of each). Compounds:

S_A 1st spot: formaldehyde ($R_f = 0.05$)
2nd spot: acetaldehyde ($R_f = 0.09$)
3rd spot: propionaldehyde ($R_f = 0.20$)

S_K (very little acetaldehyde)
very much acetone ($R_f = 0.40$)

M_E one pink spot
indicating the presence of a 2-enal compound ($R_f = 0.065$)

M_A 1st spot: small amount of acetaldehyde
3rd spot: small amount of propionic aldehyde
4th spot: iso-butyraldehyde (2-methyl-propanal) ($R_f = 0.29$)
5th spot: butyraldehyde ($R_f = 0.32$)
6th spot: iso-valeraldehyde (2-methyl-butanal) ($R_f = 0.39$)
7th spot: valeraldehyde ($R_f = 0.43$)
8th spot: caproaldehyde ($R_f = 0.52$)
9th spot: oenanthal ($R_f = 0.58$)
(the two intense spots represent iso-valeric and caproic aldehyde).
Above the spot of oenanthal there are another six reddish brown spots indicating the presence of aldehydes of high molecular weights.

M_K yellowish brown spots suggesting the presence of methyl-ketones of high molecular weights, but due to the lack of model substances these could not be identified

2.4. Thin-layer chromatography of the bis-DNPH-derivatives eluted from the zinc carbonate plate

2.4.1. Separation of the dicarbonyl-bis-DNPH-derivatives obtained from the flavour compounds formed during cooking of pork. The results obtained by means of the modified method of SCHWARTZ and co-workers (1968) (see para. 1.5.1) are shown on the chromatogram in Fig. 6.

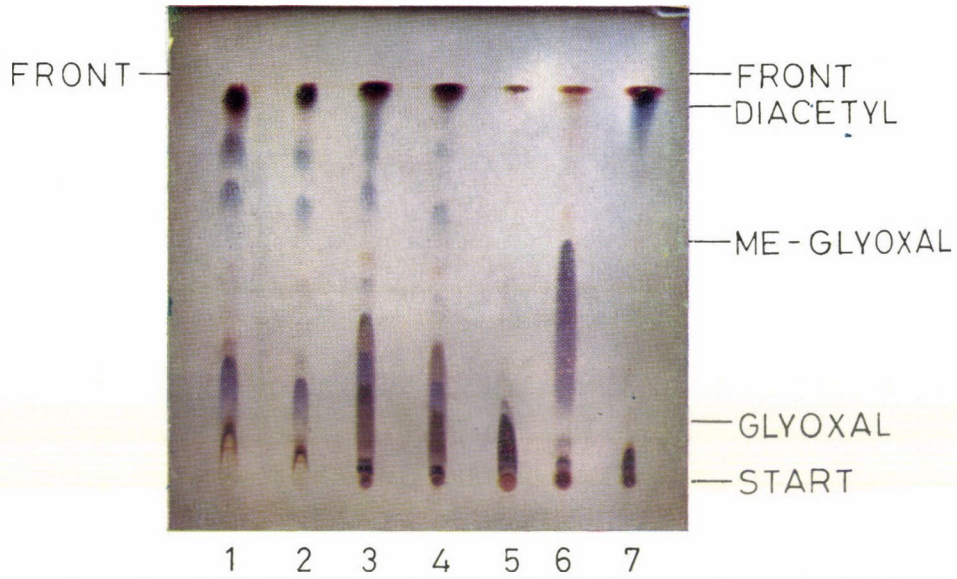

Fig. 6. Separation of dicarbonyl-bis-(2,4-dinitrophenylhydrazones) from volatiles formed during cooking of pork into alpha-dicarbonyl groups on a MgO layer. Layer: magnesium oxide. Solvent: chloroform – methanol (95 : 5). Samples: 1. dicarbonyl-bis-DNPH-derivatives from pork flavour (0.02 ml); 2. dicarbonyl-bis-DNPH-derivatives of pork flavour (0.01 ml); 3. dicarbonyl-bis-DNPH-derivatives from pork flavour (0.08 ml); 4. dicarbonyl-bis-DNPH-derivatives from pork flavour (0.04 ml); 5. glyoxal-bis-DNPH-standard (0.02 ml); 6. methylglyoxal-bis-DNPH standard (0.02 ml); 7. diacetyl-bis-DNPH standard (0.02 ml)

The chromatogram provides evidence of the presence of all three aliphatic dicarbonyl groups in the flavour of boiled pork. The spots between the starting point and the spot of the glyoxal model compound suggest that other dicarbonyl compounds might also be present.

The R_f values of the various groups of compounds are between the following limits:

- dialdehydes 0.15 to 0.58
- alpha-keto-aldehydes 0.58 to 0.86
- alpha-diketones 0.86 to 0.98.

It appears clearly on the chromatogram that both the dialdehyde and the alpha-keto-aldehyde fractions are made up of several components.

2.4.2. Separation of the dicarbonyl-bis-DNPH-derivatives obtained from the flavour compounds formed during cooking of pork. The chromatogram obtained by applying RONKAINEN's method (1967) is presented in Fig. 7.

It appears from this figure, too, that the bis-derivatives of the flavour contain all three groups (dialdehydes, keto-aldehydes and diketones) of aliphatic dicarbonyls.

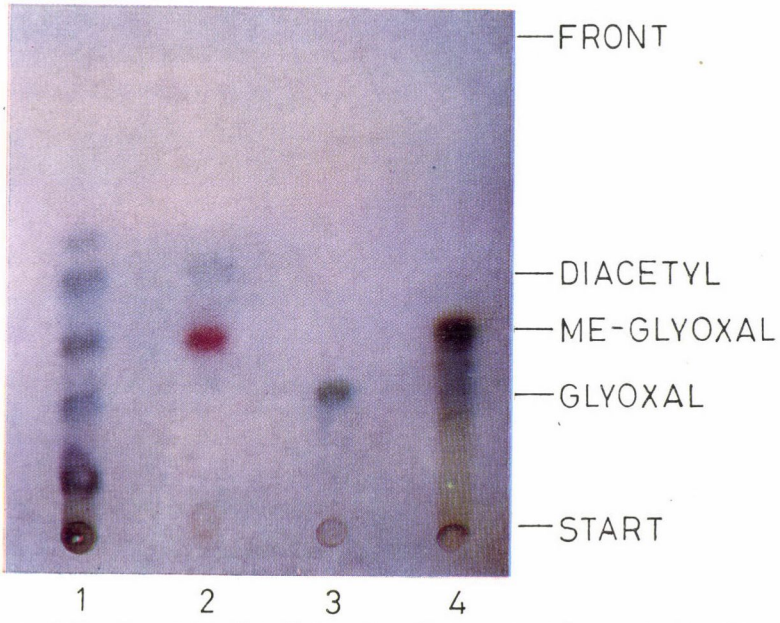

Fig. 7. Separation of dicarbonyl-bis-(2,4-dinitrophenylhydrazones) from volatiles formed during cooking of pork into alpha-dicarbonyl compounds on pre-coated Polygram Sil G plates. Layer: Polygram Sil G. Solvent: benzene – petrol ether (40–70°C) – ethyl acetate (85 : 12.5 : 2.5). Samples: 1. dicarbonyl-bis-DNPH-derivative from boiled meat flavour (0.03 ml); 2. model diacetyl-bis-DNPH-derivative standard (0.02 ml); 3. glyoxal-bis-DNPH-derivative standard (0.02 ml); 4. methylglyoxal-bis-DNPH-derivative standard (0.02 ml)

Near the start, but below the spot of glyoxal there are some intense bluish violet spots on the first chromatogram, suggesting again the presence of some other dicarbonyl compounds.

The chromatogram presents also evidence of another dicarbonyl beside glyoxal ($R_f = 0.27$), methylglyoxal ($R_f = 0.41$) and diacetyl $R_f = 0.52$ present in meat flavour. The spot of this dicarbonyl appears above that of diacetyl and might be some other alpha-diketone derivative.

2.4.3. Investigation of the aliphatic alpha-diketone homologues. The chromatogram obtained by the method described in para. 1.5.3 is illustrated in Fig. 8. It appears from Fig. 8 that among the volatile carbonyl compounds obtained from pork boiled by the method described above, altogether three

alpha-diketones, including diacetyl, could be detected. These are represented by the bluish violet spots on the chromatogram. The yellowish brown spot might be due to some impurity, probably to some monocarbonyl-DNPH-derivative. (The R_f-value of diacetyl is 0.11.)

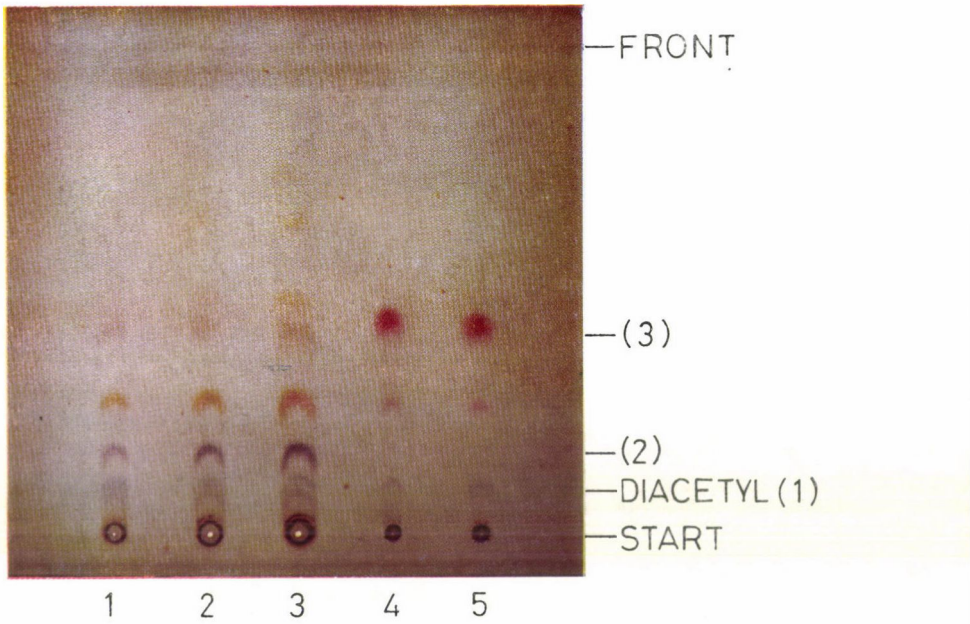

Fig. 8. Separation of the alpha-diketone-bis-DNPH fraction from volatiles formed during cooking of pork into homologues on impregnated layer. Layer: Kieselgur G impregnated with polyethylene glycol 400. Solvent: hexane – benzene (3 : 1). Samples: 1. alpha-diketone fraction from meat flavour (0.01 ml); 2. alpha-diketone fraction from meat flavour (0.02 ml); 3. alpha-diketone fraction from meat flavour (0.03 ml); 4. diacetyl-bis-DNPH-derivative standard (0.01 ml); 5. diacetyl-bis-DNPH-derivative standard (0.02 ml)

3. Conclusions

Summarizing our results we may say that in the flavour of boiled pork the quantity of monocarbonyl compounds is far greater than that of the dicarbonyls.

Of the monocarbonyls acetone, and normal alkanals from C_1 to C_7 were identified and iso-butyric and iso-valeric aldehyde were also detected. We were, however, unable to identify six aldehydes of which we know that they have carbon chains longer than C_7, one 2-enal and of the normal alkanones three unknown ketones.

Of the dicarbonyl derivatives the presence of glyoxal, methylglyoxal, diacetyl and two alpha-diketones is proven and the alpha-keto-aldehyde and dialdehyde fractions were further found to consist of several components.

We have compared the carbonyl compounds obtained from boiled pork and identified by us with similar compounds detected by other authors in various boiled meats (pork, lamb, beef and chicken). This comparison is shown in Table 1.

It appears from the table that HORNSTEIN and CROWE (1960) detected the same normal alkanes as we and identified in addition some 2-enals and 2,4-dienals too. These authors do not mention the presence of dicarbonyls, moreover much later MERRITT (1972), too, omitted their discussion.

In their investigation of the volatile carbonyl compounds from pork products (irradiated ham and fermented sausage) OCKERMAN and co-workers (1964) identified, in addition to the above mentioned monocarbonyls, diacetyl and aldol, LILLARD and AYRES (1969) diacetyl and 2,6-hexane-dione and HALVARSON (1973) only diacetyl. Neither of them found glyoxal, methylglyoxal or other dicarbonyl compounds.

HORNSTEIN and CROWE (1963) and MERRITT (1972) identified only some aldehydes and methylketone from boiled lamb, while JACOBSON and KOEHLER (1963) mention some dicarbonyl compounds in the flavour of the same meat, without identifying them.

The monocarbonyl composition of the flavour of boiled beef does not differ from the corresponding compounds of the pork or lamb flavour. Of the dicarbonyls YUEH and STRONG (1960), HIRAI and co-workers (1968, 1973) and PERSSON and VON SYDOW (1973) proved the presence of diacetyl, acetoin and 2,3-pentadione.

HIRAI and co-workers (1968, 1973), PERSSON and VON SYDOW (1973) identified some aromatic carbonyls, furane derivatives and 2-enal-derivatives.

The carbonyl compounds of chicken flavour are even more complex. Formaldehyde is missing from among the monocarbonyls. The series of dicarbonyls included 2,4-pentadione and glyoxal (MINOR *et al.*, 1965b; WILSON & KATZ, 1972). NONAKA and co-workers (1967) identified several aromatic aldehydes and furane derivatives, WILSON and KATZ (1972) succeeded, too, in detecting several types of aromatic carbonyls and a number of 2-enals and 2,4-dienals.

Thus, it appears that the carbonyl compounds of chicken flavour are the most varied.

Of the carbonyl compounds of pork flavour monocarbonyls with medium and long carbon chains might be the characteristic ones, since short chain monocarbonyls have been detected in all food flavours investigated so far.

The dicarbonyl compounds might also be typical constituents of pork flavour, since some of them are missing from many food flavours (methylglyoxal, for instance, has not been identified in any of the meat flavours).

Table 1

Volatile carbonyl compounds identified in various boiled meat flavours by different authors

Carbonyl compounds	Pork			Pork products				Lamb			Beef					Chicken					
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)
Formaldehyde	+			+	+	+		+													
Acetaldehyde	+	+		+	+	+		+	+		+	+			+	+	+	+	+	+	
Propionaldehyde	+	+		+	+	+			+			+				+		+			
i-Butyraldehyde	+			+		+									+						
Butyraldehyde	+				+				+						+	+	+	+		+	
i-Valeraldehyde	+			+		+							+		+						
Valeraldehyde	+			+	+	+	+		+				+	+	+	+	+	+	+	+	+
Caproaldehyde	+	+	+		+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
Oenanthal	+				+	+	+		+				+	+	+		+		+	+	+
Acetone	+	+	+	+		+		+		+	+	+			+	+	+	+	+		+
Glyoxal	+																		+		
Methylglyoxal	+																				
Diacetyl	+			+	+	+					+		+	+	+	+	+	+			+
2,3-Pentadione															+						+
2,4-Pentadione																			+		+
2,6-Hexadione					+																
Acetoin													+	+			+		+		+
Aromatic derivatives													+	+	+					+	+
Furane derivatives													+	+	+					+	
2-enals	+	+			+	+	+	+				+	+	+		+	+			+	+
2,4-dienals		+			+	+	+	+				+				+	+	+			+

(1) Kozma (1976) (present article)
(2) Hornstein and Crowe (1960)
(3) Merritt (1972)
(4) Ockerman and co-workers (1964)
(5) Lillard and Ayres (1969)
(6) Halvarson (1973)
(7) Watanabe and Sato (1970)
(8) Hornstein and Crowe (1963)
(9) Jacobson and Koehler (1963)
(10) Merritt (1972)
(11) Yueh and Strong (1960)
(12) Hornstein and Crowe (1960)
(13) Hirai and co-workers (1968)
(14) Hirai and co-workers (1973)
(15) Persson and Von Sydow (1973)
(16) Pippen and co-workers (1958)
(17) Pippen and Nonaka (1960)
(18) Minor and co-workers (1965a)
(19) Minor and co-workers (1965b)
(20) Nonaka and co-workers (1967)
(21) Wilson and Katz (1972)

Our method of thin-layer chromatography has the advantage of simplicity and might be applied not only to food flavours, but to the determination of carbonyl compounds in general.

*

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OCCURRENCE OF LIPOFUSCIN-LIKE PIGMENTS IN PORCINE ADIPOSE TISSUE

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It was observed that the dry cured back fat of pig sometimes turned yellow within 14–20 days giving rise to difficulties in further processing. The mechanism of discoloration, the chemical characteristics of the pigments and possibility of their separation were studied.

The yellow pigments of back fat were separated into 3 different fractions (K, M and E) by column chromatography. The fractions examined by UV, IR and fluorescence spectroscopy showed the characteristics of lipofuscin-like pigments and they were possibly formed in the following chemical process: the secondary products of fatty acids oxidation *e.g.* malonaldehyde, linked with the primary amino groups of proteins or phospholipids produce yellow *Schiff*-base type compounds.

Physiological or biochemical effects producing lipofuscin-like pigments were not investigated yet. It is supposed that two factors could be important: the deficiency in vitamin E and stress.

Yellow or brown discoloration of adipose tissues is a long-known problem. The phenomenon can generally be attributed to various causes: deficiency of natural antioxidants resulting in intensive oxidative rancidity; oxidative colour changes accompanied by pigment formation; other disorders in oxygen supply.

The oxidation processes taking place in the tissues, and particularly the autooxidation of fatty acids are especially accelerated as compared to their usual rate by using feed deficient in vitamin E. This process is often indicated by a yellow discoloration of the adipose tissues (DAM, 1944; DAM & GRANADOS, 1949). When occurring in the presence of higher amounts of unsaturated fatty acids, the discoloration can reach such an extent that the adipose tissues turn brown (ROBINSON & COEY, 1951). In the yellowing of fats caused by vitamin E deficiency, sulphur-containing amino acids and selenium complexes also play an important part (HORVÁTH & NACEV, 1972). However, the composition and structure of the coloring agents is as yet unknown (CHEN & PACKETT, 1972).

As a result of vitamin E deficiency, not only the autooxidation rate of the fats will increase, but other changes will also take place in the organism. For example, yellow or brown pigments will be deposited in the adipose tissues and in the liver. These pigments are termed lipofuscin or ceroid. (NOBORN, 1966; WEI KING, 1967). Lipofuscin has long been known in medical practice

as a by-product of cellular metabolism appearing in the older age (age-pigment). Its composition and structure has not, however, been elucidated (SEIBER & DIESEL, 1962; BJOERKERUD & CUMMIUS, 1964; DROZD, 1972). Lipofuscin is also formed in younger cells when disorder in the oxygen supply of the organism occurs (BJOERKERUD, 1963; KUMAMATO & BOURNE, 1963; HENDLAY & STREHLER, 1965). Deposition of lipofuscin has been observed in the liver (BERNEIS & STUDER, 1968; OHANO & MATSUMATO, 1960), in the heart (HEIDENREICH, 1965; CLEMENTE & CORDIANI, 1968), in the nervous tissues (HUTTNER & HARANGHY, 1966; BARKA & SCHENER, 1964), in the kidneys (WINTER, 1963; HARRIS, 1966) and in the adipose tissues (KENKICHI & KAZDHO ISHIDA, 1958).

Lipofuscin was also found in the adipose tissues (MASORO, 1969; NIKODIJEVIC & KOVACEV, 1972) when, as a result of a stress state owing to various causes, important changes took place in fat metabolism, *e.g.* free fatty acids had been mobilized (HAYES & NIELSEN, 1969; STUDER & SCHAERER, 1965). This discoloration is sometimes termed "fat stress phenomenon" (HAYES & NIELSEN, 1969).

Several researchers describe lipofuscin-like pigments, which — though very similar to lipofuscin in properties — differ from the latter in their formation process (SAMOROJSKI & KEEFE, 1964; TAPPEL *et al.*, 1969). Thus *e.g.* peroxides formed in the autooxidation of unsaturated fats will rapidly decompose into compounds containing carbonyl groups, *e.g.* malonic aldehyde. In turn, the latter are capable of reacting with the primary amino groups of proteins or phospholipids, yielding yellow or brown polymers of the *Schiff*-base type (TAPPEL *et al.*, 1969).

Since the fluorescence spectra of these products are very similar to that of lipofuscin, the yellow polymers were termed *lipofuscin-like pigments*. Feeding with fodder deficient in vitamin E largely promotes the formation of lipofuscin-like pigments (TAPPEL *et al.*, 1973).

POKORNY and JANICEK (1973b) prepared yellow or brown polymers in model systems consisting of lipids with different degrees of unsaturation and albumin. The formation process was identical with that of lipofuscin-like pigments. However, no data on the fluorescence of the synthetic pigments were published.

So the term lipofuscin should rather be considered a collective term for all fat-soluble pigments formed in various processes.

During the last few years, it was observed that some of the processed adipose tissues of pig (back fat), in the dry salted state, turn yellow in about 14 to 20 days. The extent of yellowing largely differs from batch to batch. The usual veterinary control of the animals did not indicate any pathological changes. An examination of rancidity characteristics demonstrated that the yellow discoloration is not simply due to oxidative rancidity. This is also

confirmed by the fact that all other organoleptic characteristics of the yellow fat remained unchanged.

It was attempted to attribute the discoloration of the pig back fat to one of the causes discussed in the foregoings.

1. Materials and methods

1.1. Sampling

We chose 10 samples of back fat from animals whose breed, sex, age and fodder composition was known (Table 1; Table 1/a).

1.2. Storage

The samples were dry cured and stored at +2 °C. The amount of NaCl was 10% on the weight of the sample.

Table 1

Characteristic data of the investigated pig back fats

Sample No.	Breed	Sex	Age (days)
1	UL×H	♂	219
2	U×L	♂	228
3	U×L	♂	221
4	U×L	♂	228
5	UL×L	♂	215
6	U×L	♂	221
7	U×L	♀	219
8	UL×H	♂	234
9	UL×L	♀	215
10	UL×H	♀	223

U = Hungarian White
H = Hampshire
L = Dutch Flatland

Table 1/a

Fodder composition

Maize (%)	Wheat (%)	Soya (50%)	Biolysine (%)	AP-14 (%)	Salt (%)	Fodder lime (%)	Premix* (%)
10	14.2	22.0	0.4	0.5	0.4	1.0	0.5

* According to Hungarian Standard

1.3. Determination of vitamin E

The total percentage of tocopherol in the samples was determined spectrophotometrically, using the *Emmerie–Engel* reaction (BERNDORFER–KRASZNER, 1966).

1.4. Determination of fatty acid composition

This was carried out by gas chromatography; 1.0 g of the sample was extracted for 4 hours with about 50 ml diethyl ether, using the *Besson* method. After removal of the solvent, 15 ml of 0.5 *M* methanolic KOH were added to the residue and the mixture was boiled for 10 min to obtain satisfactory saponification. 5 ml of a 10% methanolic BF_3 solution were then added. The acid solution was boiled for a further 3 min, 10 ml of n-hexane were added, the heating was discontinued and the mixture was allowed to boil for a further min. It was then cooled and 10 ml of methyl benzoate were added as reference standard.

Subsequently 30 ml of distilled water and 5 g NaCl were added, the mixture was shaken until the salt was dissolved, and transferred into a separating funnel. The separated organic phase containing the fatty acid esters was stored over anhydrous Na_2SO_4. Two to 5 μl of this solution were used in the tests. Gas chromatography was carried out using *Chromosorb* W (product of CARLO-ESTE) as support, 10% polyethylene-glycolsuccinate polyester as distributing liquid (product of SERVA), in nitrogen stream. The temperature of the evaporating space was 230 °C, that of the column 180 °C. A *Chrom-3* IK 2 type instrument and a flame ionization detector were used.

1.5. Identification of lipofuscin

Lipofuscin was determined in the yellow samples by a histochemical method (KRUTSAY, 1971). These experiments were carried out in the PATHOLOGICAL HISTOLOGY DEPARTMENT OF THE NATIONAL TRAUMATOLOGICAL INSTITUTE Budapest.

1.6. Tests of yellow coloring agents

1.6.1. Thin-layer chromatography. The presence of colored substances in the samples was demonstrated by the thin-layer chromatographic method reported by POKORNY and JANICEK (1973a).

1.6.2. Recovery and separation of the pigments. The discolored fat sample was minced and homogenized. Twenty g of the homogenized mass were weighed into an Erlenmeyer flask and 100 ml of petrol ether were added. The mixture was agitated for 1 hour and filtered through a folded filter. After

the evaporation of the solvent a yellow, readily melting fat was obtained, from which the pigments could be isolated by purification through a chromatographic column.

To 2 g of the fat obtained by extraction, 50 ml of an 0.5 *M* methanolic KOH solution were added and the mixture was saponified by heating for 10 min on a water bath. (In this process, part of the fatty acids became water-soluble, but the pigments did not deteriorate.) The saponified mixture was then extracted 3 times with 20 ml of chloroform. The organic phase was washed with about 500 ml of water and dried by filtering through anhydrous Na_2SO_4. The yellow chloroform solution was evaporated to a volume of ca. 10 ml.

Ten g of diethyl-aminoethylcellulose (DEAE) adsorbent were washed with 100 ml of concentrated acetic acid and dried at 100 °C. The adsorbent was introduced into a 30 ml chromatographic column and saturated with chloroform. The chloroform solution obtained after saponification (10 ml) was then introduced into the column. The chromatographic procedure was started with chloroform and the eluate was collected in a 100 ml Erlenmeyer flask.

Elution was continued until 60 ml of eluate were collected. Then chromatography was discontinued and another Erlenmeyer flask was placed under the column. Chromatography was then continued with a 9 : 1 mixture of chloroform and methanol. The flask was again changed and elution continued with chloroform and acetic acid, until the eluate volume again reached 60 ml.

The solutions thus obtained were then evaporated. From the chloroform eluate, a yellow oil was obtained (fraction K). Evaporation of the chloroform–methanol eluate yielded a yellowish-brown, viscous product (fraction M), while a dark brown solid substance was obtained from the chloroform–acetic acid fraction (fraction E).

1.6.3. UV spectrophotometry. UV absorption spectra of the colored substances were measured using a MOM 204 spectrophotometer, in chloroform-containing solution in the 230–330 nm range.

1.6.4. IR spectrometry. IR absorption spectra were measured between two KBr plates, using an UR-20 spectrometer.

1.6.5. Fluorescence spectra. Fluorescence spectra were taken in chloroform-containing solution, using a *Fluorimeter* EEL 224 instrument. Chloroform freshly distilled in a glass apparatus was used. The wavelength for excitation was 380 nm, and the spectra were measured in the 400–500 nm range.

2. Results

In studies on yellow discoloration of pig fat, first the relationship between vitamin E content and fatty acid composition of the adipose tissues was determined (Table 2). Results demonstrated that — although feeding was iden-

Table 2

Vitamin E content, fatty acid composition

Trial	T_1	T_2	Yellowing	Total fat (%)	Ratio of unsaturated to saturated fatty acids
	mg/100 g				
1	1.83	0.73	±	93.73	1 : 1.28
2	1.30	0.55	—	96.46	1 : 1.71
3	0.97	0.12	—	93.55	1 : 1.66
4	0.82	0.65	±	97.72	1 : 1.49
5	0.50	0.45	+	95.14	1 : 1.87
6	0.48	0.47	+	89.05	1 : 1.33
7	0.40	0.32	±	96.15	1 : 1.21
8	0.27	0.26	—	96.35	1 : 1.60
9	0.25	0.12	+	97.10	1 : 1.39
10	0.14	0.12	±	97.74	1 : 1.68

T_1 = Total tocopherol content after slaughtering
T_2 = Total tocopherol content after two weeks storage
* Data are mean values of three parallel determinations

tical – important differences existed among the total tocopherol contents of individual samples. The percentages of unsaturated fatty acids also vary. The large differences between linolic acid percentages are particularly striking. However, the results do not unequivocally confirm the assumption that yellowing of the samples is caused by their low vitamin E content and high unsaturated fatty acid content.

No strict correlation exists between the percentages of vitamin E and of unsaturated fatty acids: the linear correlation coefficient was found to be –0.12. It could thus be ascertained that the yellow discoloration of the fat samples is not caused by an unusually rapid autoxidation of the fatty acids. It may, however, be assumed that this process is also one of the components leading to the discoloration of adipose tissues.

Next it was attempted to confirm the presence of lipofuscin pigments in the samples. Histological investigations demonstrated that color-formation is caused by a moderate amount of fat-soluble yellowish-green pigment-particles that are, however, not identical with either lipofuscin or other pigments known up to the present (JÓZSA, 1974).

The next approach aimed to explain yellowing of pig back fat by the formation of lipofuscin-like pigments. It was, therefore, attempted to demonstrate the presence of yellow polymers formed by the mechanism presented in Fig. 1, using thin-layer chromatography (POKORNY & JANICEK, 1973b).

*and discoloration data of the samples**

Distribution of fatty acids						
14 : 0 (%)**	16 : 0 (%)	18 : 0 (%)	16 : 1 (%)	18 : 1 (%)	18 : 2 (%)	18 : 3 (%)
1.30	25.46	14.20	4.20	37.86	10.65	0.022
1.40	22.55	11.59	2.60	46.98	8.82	0.025
0.87	22.73	11.70	3.63	41.82	13.30	0.020
1.37	24.17	14.34	3.29	44.51	10.00	0.021
1.56	18.02	13.53	5.11	38.62	18.26	0.012
1.41	26.62	13.62	1.90	35.87	9.51	0.009
10.93	21.20	11.30	3.53	44.92	4.23	0.011
1.80	22.45	12.66	4.14	42.20	13.05	0.022
2.24	25.48	12.83	2.01	43.51	10.97	0.033
0.99	21.70	13.78	5.01	46.44	9.77	0.042

** Percentages are related to the sample weight
$s_{Tocopherol}$ = 0.05 mg/100 g (standard deviation)
$s_{Fatty\ acid}$ = 0.5% (standard deviation)

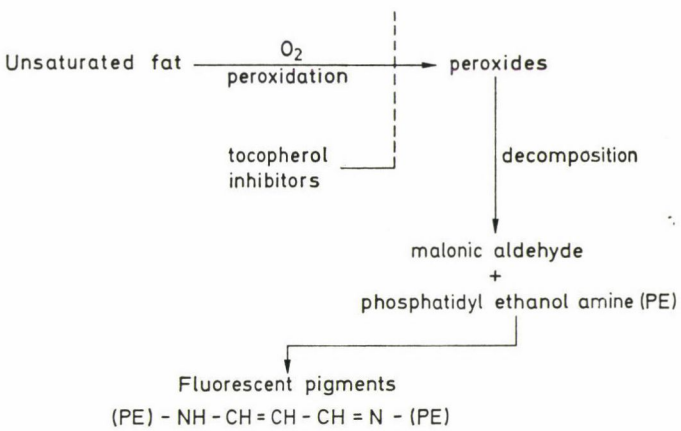

Fig. 1. Formation process of lipofuscin-like pigments

These studies confirmed that the samples contained colored substances formed in the polymerization process which resulted from the primary oxidation stage (Fig. 2).

As described in the experimental part, three different colored substance fractions (fractions K, M and E) were isolated from the discolored samples by column chromatography.

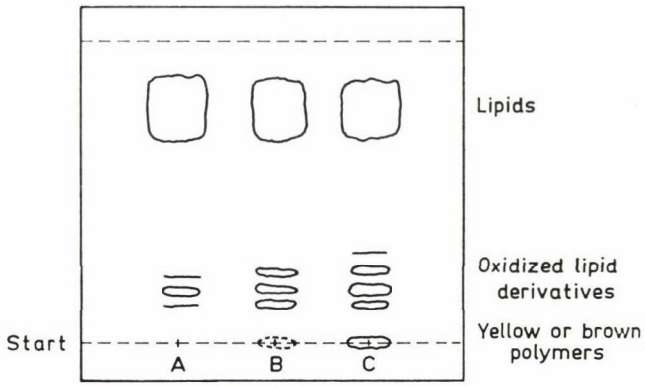

Fig. 2. Detection of polymers by thin-layer chromatography. A: Back fat samples 24 hours after slaughtering. B: Salted back fat sample after 2 weeks of storage. C: Yellow salted back fat sample after 2-weeks storage. Solvent: Benzene–acetone–acetic acid 95 : 5 : 1. Adsorbent: Kieselgel G. Developer: a 20% alcoholic solution of phosphomolybdic acid

Table 3 shows the nitrogen content of these fractions, using the micro-*Kjeldahl* method.

The nitrogen content of the fractions K and M are of the same order of magnitude as that of the polymers prepared by POKORNY & JANICEK (1973b).

Table 3

Nitrogen content of yellow pigments formed at the oxidation of fats

Sample	Nitrogen content, %
According to POKORNY and JANICEK (1973b)	0.4–1.0
Fraction K	0.60
Fraction M	0.82
Fraction E	2.80

In contrast, fraction E contains a relatively high amount of nitrogen, so that it may be assumed that a higher amount of protein participates in its formaton.

Subsequently, the pigments were identified by means of their absorption and fluorescence spectra.

The *UV spectra* of the pigment fractions are presented in Fig. 3. A very characteristic maximum is observable in the spectra of fractions K and M at 246 nm and 247 nm, resp. Another low-intensity absorption peak is present in the spectrum of fraction M at 310 nm. The UV absorption spectrum of fraction E shows three relatively low-intensity peaks at 247, 270, 340 nm.

IR absorption spectra allow to state (Figs. 4–6) that the isolated pigments consist of moderate chain-length unsaturated fatty acid esters (–C–O–C– bands appear at 1243–1248 cm^{-1}, the carbonyl band at 1 758 cm^{-1} and the unsaturated carbon bond band at 3015 cm^{-1}).

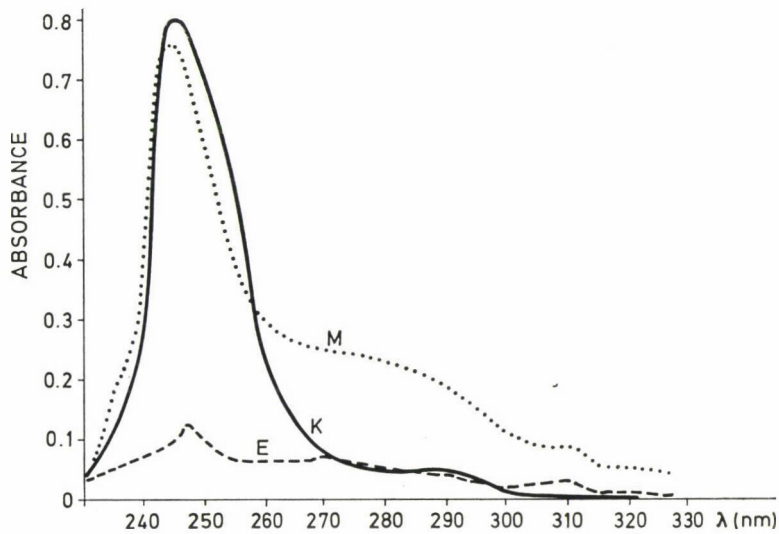

Fig. 3. UV spectrum of the colored substance fractions

Presumably fraction K consists chiefly of fatty acid esters, since no peaks corresponding to primary and secondary amino groups are present in its IR spectrum. Tertiary amino groups might be present in this fraction, but their absorption bands could not be identified in the IR spectrum.

Fractions M and E also contain –OH groups (signal at 3 600–3 400 cm^{-1}) and =NH– and $-NH_2$ groups (signal at 3 200–3 400 cm^{-1}). It may therefore

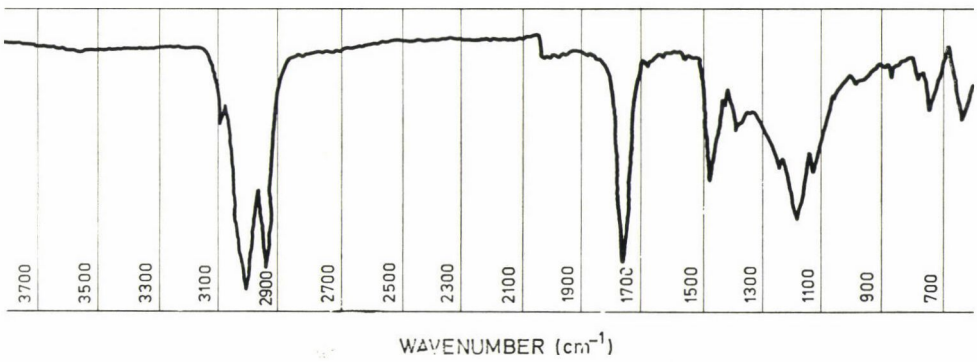

Fig. 4. IR spectrum of fraction K

be assumed that phospholipids and/or proteins participate in the formation of these fractions.

Studies of the fluorescence of these pigments further confirmed these statements, since TAPPEL and co-workers (1968, 1973) reported the characteristics of the fluorescence spectra of lipofuscin-like pigments so that the pigments isolated by us could be identified.

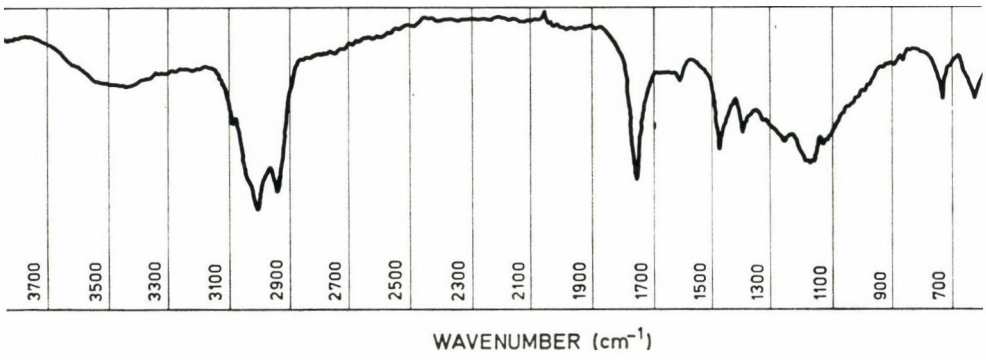

Fig. 5. IR spectrum of fraction M

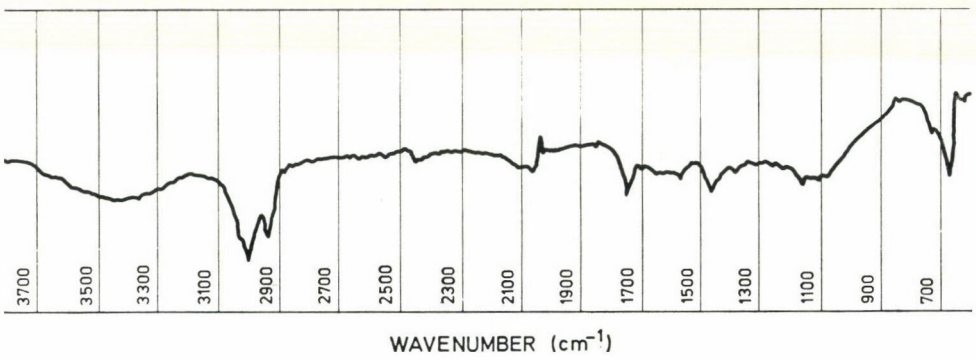

Fig. 6. IR spectrum of fraction E

The fluorescence spectra taken at an excitation wavelength of 380 nm demonstrate the presence of an intense maximum for fractions K and E and of a weaker maximum for fraction M (Fig. 7). The maximum is located at 460 nm, 430 nm and 455 nm for fractions K, E and M, resp. This is in good agreement with the data already published by TAPPEL and co-workers (1969, 1973), according to which maximum fluorescence for lipofuscin-like pigments is in the range of 450–470 nm at excitation wavelengths of 360–390 nm. (The characteristic wavelength of the autofluorescence of lipofuscin is also 460 nm.)

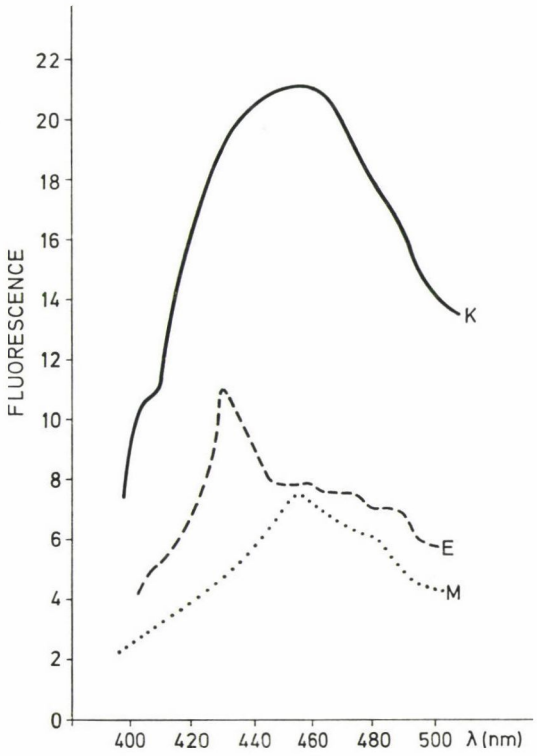

Fig. 7. Fluorescence spectra of the colored substance fractions. Excitation: 380 nm

3. Conclusions

The yellow discoloration of the dry cured back fat of pig is a great problem in industrial practice, and — in particular — is a source of important financial losses, since the discolored fat can not be used for consumption. However, very few references to a similar industrial occurrence could be found in the available literature. This can partly be explained by the fact that the processing of the raw back fat in the dry salted state to prepare cured or smoked bacon at a later time is the usual process applied in Hungary. Although smoked bacon is also being manufactured in some other countries, *e.g.* in Czechoslovakia, in Poland and in the Soviet Union, technologies corresponding to the dietary habits of these people are applied, and substantially smaller quantities are produced than in Hungary.

Our work allowed to state that the discoloration of pig back fat is caused by the formation of yellow or brown pigments. By the new separation method reported, three different fractions of pigment (K, M and E) could be isolated. The results of UV, IR and fluorescence spectra show that all three pigments consist of moderate chain-length unsaturated conjugated fatty acid esters.

Fractions M and E presumably also contain –OH and nitrogen-containing groups which could be –NH–NH–, –NH–CH=, –N=N– or $-NH_2$ groups. However, the location and number of these groups, *i.e.* the exact structure of the pigment fractions cannot be determined on the basis of UV and IR spectra. The absorption bands characterizing these groups have not yet been published since the location of the absorption maximum largely depends on the degree of association and on the structure of the molecule.

Analysis of the spectra and thin-layer chromatographic studies demonstrated that the pigments isolated from the adipose tissues are identical with or very similar to the lipofuscin-like pigments already published (TAPPEL *et al.*, 1969; POKORNY & JANICEK, 1973a). Hence, these pigments are presumably formed by the chemical reactions presented in Fig. 1: one of the major products of unsaturated fatty acid peroxidation, *viz.* malonic aldehyde, reacts with lipids or with the free amino groups of phospholipids or proteins. As a result, products of the *Schiff*-base type, containing several chromophores, are formed of characteristic fluorescence maxima at 450–470 nm (with excitation wavelengths of 360–390 nm).

On the basis of the experimental results, it may be assumed that, among the pigments isolated from the yellow adipose tissues, fraction K is mainly the product of the reaction between malonic aldehyde and lipids. Phospholipids are probably reacting with malonic aldehyde to yield fraction M, while the reaction of proteins with malonic aldehyde appears to be responsible for the formation of fraction E. Summing up the results, we may state that the yellow discoloration of pig adipose tissues is caused by the presence of lipofuscin-like pigments.

In the knowledge of the formation process of lipofuscin-like pigments, it becomes understandable that the peroxide number and acid number of the discolored samples were found to be small, and their *Kreiss* reaction was negative, since these characteristics indicate the amounts of primary oxidation products (peroxides) and secondary oxidation products (acids, aldehydes).

The presence of lipofuscin-like pigments is also confirmed by the experience that no lesions were observed in the slaughtered animals in normal veterinary control, since in general these pigments are formed as a result of some disorder in the oxygen supply of the organism, which, however, cannot or can hardly be observed in their early stage.

However, these results do not satisfactorily explain why the formation of lipofuscin-like pigments takes place so rapidly, and why intense discoloration appears only with some of the samples. In fact the conditions necessary for the reaction of pigment formation *e.g.* peroxides from unsaturated fatty acids, malonic aldehyde or some other compounds containing a carbonyl group, phospholipids and proteins are present in all adipose tissues.

It, therefore, appears probable that the animals whose adipose tissue

turns yellow during storage have been subjected to such environmental or biochemical impacts that start or catalyze pigment formation. Based on data already published and on the present experimental findings, major importance should be attributed in particular to two factors, *viz.* to the changes in vitamin E levels and to the stress effects provoked by the environment.

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HYDROGENATION USED IN THE DETECTION OF ZEARALENONE (F_2 TOXIN)

PRELIMINARY COMMUNICATION

I. SARUDI, JR.

(Received April 15, 1975)

The 4-methoxy-benzene-diazonium-fluoroborate method may be used in detecting F_2 toxin after separation by thin-layer chromatography even upon hydrogenation of its oxo-group with sodium-borohydride in a methanol-containing medium. However, a substantial difference is observed between the R_f-values of the original toxin and its transformed variant. This serves as a valuable information in the detection of F_2 toxin.

It was shown in an earlier communication (SARUDI, 1974) that F_2 toxin produced by certain *Fusarium* strains, may be detected by the 4-methoxy-benzene-diazonium-fluoroborate method. In the present study the method was made more specific by utilizing the oxo-group of the compound beside the structural characteristic as mentioned earlier. The essential feature of the method is the selective hydrogenation of the oxo-group in the molecule with sodium borohydride thereby producing an alcoholic hydroxyl radical (HODGE *et al.*, 1966).

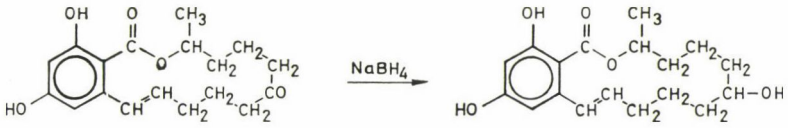

Fig. 1. Selective hydrogenation of the oxo-group in the F_2 molecule with sodium boro hydride

Under appropriate thin-layer chromatography conditions this modification causes a significant change in the R_f-value and thus serves with valuable information in the detection of the F_2 toxin.

The F_2 derivative produced by selective hydrogenation was earlier investigated by MIROCHA and co-workers (1967). In the course of their research into the molecular structure they examined the compound by various methods of spectroscopy.

1. Materials, methods and results

1.1. Materials

Petroleum ether (B.p. 40–70 °C), chloroform; methanol; 95% ethyl alcohol; 95% acetic acid; Kieselgel G (Stahl); sodium borohydride; methyl alcohol solution containing 50 ppm F_2 toxin, using crystalline F_2 preparation. Detecting reagents: 0.5% 4-methoxy-benzene-diazonium-fluoroborate solution; 0.1 *M* alcoholic KOH solution; 1 : 1 mixture of conc. H_2SO_4 and 95% ethyl alcohol.

1.2. Hydrogenation of the oxo-group of F_2 toxin and some characteristics of the reduced compound

Ten ml of the methyl alcoholic F_2 solution were transferred into a 50 ml beaker and very small portions of sodium borohydride were added up to a total of about 0.1 g. (A further portion of the reagent was added when gas formation nearly ceased.) After this step, taking about 10–15 min, the super-

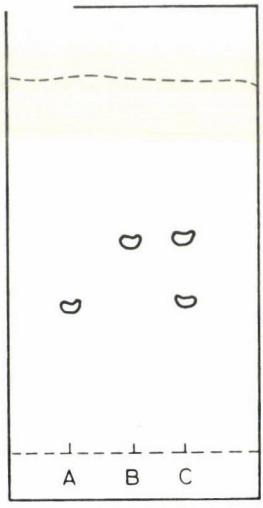

Fig. 2. Thin-layer chromatogram of the F_2 toxin with hydrogenated oxo-group ("A") and of the original F_2 toxin ("B") and of the mixture of the above two compounds. Adsorbent: Kieselgel G (Stahl); developing agent: chloroform : 95% ethyl alcohol, 95 : 5

fluous reducing agent was degraded by adding 3–4 drops of conc. acetic acid. The solution was then nearly evaporated over a water bath. One ml of methyl alcohol was added to the residue (solution "A"). This solution contained some salt poorly soluble, however, this did not affect the process.

The reduced F_2 derivative was then applied to a thin-layer. For the sake of comparison two further solutions were applied to the thin-layer: the ten-

fold concentrate of the original F_2 solution, containing 50 ppm of this compound (solution "B"), and the 1 : 1 mixture of solutions "A" and "B" (solution "C"). Of each of the three solutions 0.05 ml were applied. The compounds were run on Kieselgel G (Stahl) layer in chloroform–95% ethyl alcohol mixture (95 : 5). The final distance of the solvent front from the start line was about 150 mm. After development the thin-layers were dried at room temperature.

The F_2 derivative obtained by reduction was shown to have similar fluorescence characteristics, under UV light, to that of the original F_2 toxin, while the colour reaction of the two compounds with 4-methoxy-benzene-diazonium-fluoroborate was exactly the same. The spot of the derivative, however migrated at a speed different from that of the original compound: the R_f value of the hydrogenated compound was 0.4, while that of the original 0.55.

1.3. Detection of the toxin in animal feed

Eighty g of the finely ground sample (corn, wheat, mixed feed, *etc.*) were weighed in a 500 ml iodine determination flask, 150 ml petroleum ether were added and after shaking by machine for 5 min the solution was filtered through a folded filter. After evaporating the solvent the residue on the filter was placed in a 500 ml iodine flask, 150 ml chloroform were added, and the mixture was shaken for 2 hours. Then it was again filtered through a folded filter and the solute evaporated over a water bath to syrup thickness. This residue was then diluted in 10 ml methyl alcohol and a 5 ml aliquot transferred to a test tube. The aliquot was then hydrogenated according to para 1.2 and the content of the test tube was then mixed with the substance not hydrogenated. (This substance corresponded essentially to solution "C".) The solution was evaporated to some tenth of a ml. This residue was applied to the thin-layer. The chromatogram was developed according to para. 1.2. If the feed contained F_2 toxin two spots of similar colour were seen on the layer, one at $R_f = 0.4$ and the other at $R_f = 0.55$.

By hydrogenating the oxo-group of F_2 toxin information on the compound was increased and thereby the reliability of detection was improved. This method proved particularly useful with mixed feeds where the spot of F_2 was covered by the spot of another component.

2. Conclusions

For the lack of appropriate experimental material the problem, whether the method is suitable in case of samples containing compounds related to F_2, could not be investigated as yet.

*

Thanks are due to Dr. M. PALYUSIK, candidate of the veterinary sciences, for placing the purified, crystalline F_2 compound at our disposal.

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A STUDY OF THE HOWARD COUNT OF TOMATO PULP WITH A VIEW TO ITS USE IN THE CONTROL OF PLANT HYGIENE

I. LENDVAI, Á. BOLDOG-GYŐRI, I. FÁBRI and I. KARDOS-HUSZÁR

(Received May 15, 1975)

One of the characteristics of the microbiological quality of tomato products is their mould count or Howard count. This permits of drawing conclusions as to the quality of the raw material and to plant hygiene practices. Testing fresh pulp immediately upon pulping may be considered one of the critical points of in-line control. The authors, on the basis of analyses extending over several years, found a good correlation between the Howard mould count of the fresh pulp and that of the final product. The aim of the study was to establish Howard count limits for the pulp which secure an acceptable mould count in the final product (concentrate). Year by year data were evaluated by two-variable regression analysis. Supported by statistical tests a correlation of general validity between the Howard count of fresh pulp and the final product was established. On the basis of these calculations it is possible to determine the recommended Howard count limit of the pulp to produce a concentrate of the required Howard count. The method may also be used to control plant hygiene practices, and thereby to discover defects in handling of the raw material, *e.g.* in transport, storage or sorting, *etc.*

The quality and storage characteristics of foods are highly dependent on the observation during production for the requirements of plant hygiene. One of the most efficient methods for the control of plant hygiene is in-line testing of microbial contamination. The efficiency of this test depends partly on the knowledge of the critical points of processing and partly on the knowledge of the microorganisms affecting mostly the quality of the product and the permissible limit values.

Tomatoes are prone to rapid deterioration, in particular to moulding, therefore, as an indicator of their microbiological quality, the mould count was introduced by HOWARD and STEPHENSON (1917). TROY (1956) established a correlation between the Howard count of the final product and the degree of deterioration of the raw tomatoes. According to his observations if the mouldy parts exceeded 2% (w/w) of the raw tomatoes the Howard count of the final product will reach 40%. It is an accepted practice in international trade among tomato processing plants and customers of tomato products to consider the Howard mould count as the index of the microbiological quality of raw tomatoes and of plant hygiene. In tomato processing the critical point to check the conditions of microbiology seems to be the control of the pulp. The raw tomato pulp may be considered a homogeneous intermediary product suitable for

testing without preliminary preparations. Since pulping is the central operation in tomato processing, the mould count of the pulp permits of drawing conclusions as to the hygiene of raw material handling, on one hand, and to the quality of the concentrate, on the other. However, a basic condition of this is the establishment of a correlation between the Howard count of the fresh pulp and that of the final product. In order to be able to analyse the correlation, the error inherent in the Howard count determination method has to be known. On the basis of 1 500 data, VAS and co-workers (1959) determined the error of the Howard count method for tomato purée samples of varying mould content.

The aim of this study was to find out whether it was possible to draw conclusions from the Howard count of the raw pulp as to that of the paste. The study was based on the mathematical statistical evaluation of the great number of Howard counts collected during several years in the BÉKÉSCSABA CANNING FACTORY. Further an assay was made to establish the limit value of Howard count in the pulp that permits of a suitable final product. Since the strict standards for the final product specify 40% or 50% as the upper limit of acceptance, it seemed expedient to establish two limiting values for the pulp, too.

1. Materials and methods

In this investigation the Howard counts of about 450 tomato paste batches, manufactured during the 4 years from 1970 through 1973 in the BÉKÉSCSABA CANNING FACTORY, were evaluated. For each batch the Howard count of the pulp and that of the paste was determined. Tomato paste manufacture is a continuous process in the course of which concentration takes 6 hours. Thus, to handle separately the experimental batch the paste sample was taken 6 hours after the sampling of the fresh pulp. Two kinds of paste were studied: one of refractive index 28–30%, and the other of 38–40%.

The Howard count determination method used was as laid down in the AOAC description (1970).

The mathematical statistical evaluation was carried out as follows (WEBER, 1971). The frequency distributions of the Howard counts of the 450 batches were plotted in histogrammes, one for the fresh pulp and one for the paste.

In order to establish the correlation between the Howard counts of the pulps and those of the concentrates two-variable linear regression analysis was carried out. Every year the Howard counts of the final products (C) were plotted against the Howard counts of the pulps (P). To obtain a regression equation of general validity, independent of the variations in yearly data,

3 basic statistical tests were carried out. The homogeneity of standard deviation about the regression curve (s_{CP}) was checked by *Bartlett*'s test.

A basic condition of the determination of the coefficient (b) of the regression curve of general validity is that the coefficients (β) belonging to the regression equations of the Howard counts of individual years should not differ to a significant degree from the general regression coefficient, that is the following requirement shall be fulfilled:

$$b - SD_b \leq \beta \leq b + SD_b$$

where SD_b stands for the significant difference between b and β.

First the standard deviation of the regression coefficient was determined.

$$s_\beta = \frac{s_{CP}}{s_P} \sqrt{\frac{1}{n-1}}$$

where s_β = standard deviation of the regression coefficient
s_{CP} = standard deviation about the regression curve
n = number of data used to calculate the curve
s_P = standard deviation of P values.

The homogeneity of regression coefficients was determined according to HALD (1962).

To calculate the significant difference SD_b between data of β and the mean values (b) by means of the average standard deviation ($\bar{s}_\beta = s_b$), the t test was used

$$SD_b = s_b \cdot t$$

where SD_b = significant difference between values b and β
s_b = mean of the values s_β
t = appropriate value of t test.

To determine the other parameter of the general regression curve, the C values marked by C', belonging to the average P value relevant to all the batches (Howard counts of the pulp) for the individual regression curves, were calculated. The C' values were then tested for significant difference, or whether the following requirement was fulfilled:

$$SD_{\overline{C'}} \leq C'_i - C'_{i+1} \qquad \text{where } i = 1 \ldots 5.$$

The significant difference was calculated, taking into account the average s_{CP} value, by the t test:

$$SD_{C'} = t \cdot s_{CP} \sqrt{\frac{2}{\tilde{n}}}$$

where $SD_{C'}$ = significant difference between values C'
$\tilde{n}$ = harmonic average of data.

On fulfilling the requirements the regression equation of general validity was calculated:

$$C = \bar{C} + b(P - \bar{P})$$

where $\bar{C}$ = average of values C'
$\bar{P}$ = average of values P
b = average of values β

The boundary Howard count values of the pulp belonging to the specified Howard counts for paste were determined, taking into account the confidence interval, using general regression curve.

2. Results

Fig. 1 shows the relative frequency distribution of the Howard counts in the pulps and in the pastes for the batches under investigation.

The regression data of Howard counts of the pastes for each year are given in Table 1.

Calculation of the general regression curve: the average standard deviation based on the s_{CP} values in Table 1:

$$\bar{s}_{CP} = 5.9$$

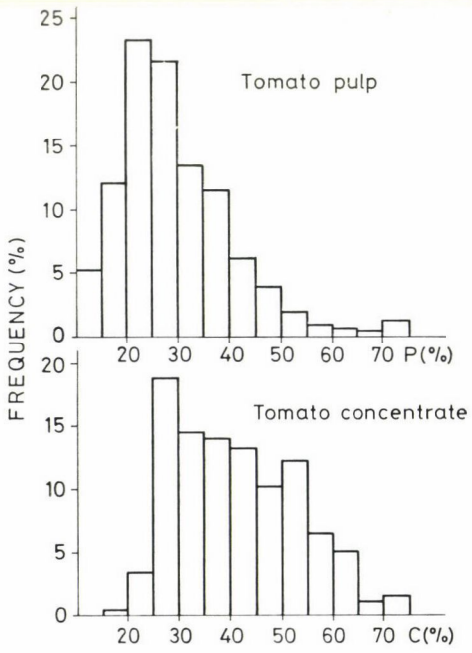

Fig. 1. Relative frequency of the Howard count data in the tomato pulp and in the tomato concentrate (1970–73)

Table 1

Correlation between Howard counts of tomato concentrates (C) and of pulps (P)

Total soluble solids of the concentrate (%)	Year of production	Regression equation (C = a + βP)	s_P	s_{CP}	r	n	C'
28	1970	C = 8.67 + 1.44 P	14.88	5.49	0.969	54	51.1
	1971	C = 3.74 + 1.33 P	16.89	6.06	0.967	180	43.0
	1972	C = 7.43 + 1.27 P	7.67	5.25	0.882	65	45.7
38	1970	C = 9.58 + 1.28 P	6.49	4.21	0.897	19	47.3
	1971	C = 16.55 + 0.91 P	9.22	6.26	0.804	61	43.4
	1972	C = 13.53 + 1.05 P	8.76	6.71	0.811	71	44.7
Overall equation of the regression line		C = 9.4 + 1.2 P	12.56	5.90		450	44.8

Symbols:

s_P = standard deviation of the Howard counts of tomato pulps (P)
s_{CP} = standard deviation about the regression curve
r = correlation coefficient
n = number of batches examined
C' = Howard count of concentrate, calculated from the average Howard count of the pulps

Derived on the basis of *Bartlett*'s test $\chi^2 = 8.5$, from the table $\chi^2 = 11.1$, therefore at $p = 95\%$ the populations studied are of identical standard deviation.

Statistical test used to calculate b:

$s_b = 0.083 \qquad t = 2.01 \qquad p = 95\% \qquad \text{D.F.} = 49$

$SD_b = 0.20 \qquad \bar{\beta} = b = 1.19$

The requirement:

$$0.99 \leq \beta \leq 1.39 \ .$$

β values contained in Table 1 do not fulfil the requirement in two cases only. However, since the two extreme β values deviate from the mean in positive and negative direction to the same extent, no importance was attached to these deviations. Their role in deducing the mean values is not significant.

The individual C' values belonging to the average P values (29.5) are shown in Table 1.

Calculation of SD_C:

$$s_{CP} = 5.9 \qquad t = 2.01 \qquad p = 95\% \qquad \text{D.F.} = 49$$

$$SD_C = 2.01 \cdot 5.9 \sqrt{\frac{2}{49}} \simeq 2.4$$

As seen from the table only value C', for the year 1970, differs to a significant extent from the rest, thus this was excluded when deducing the mean.

Based on the above the equation of the regression curve:

$$C = 44.8 + 1.2(P - 29.5)$$

$$C = 9.4 + 1.2P$$

The overall regression line is shown in Fig. 2. Considering that values used in practice approximated the average, the zone of confidence interval was drawn at $\pm s_{CP}$ distance parallel to the regression curve. Within the con-

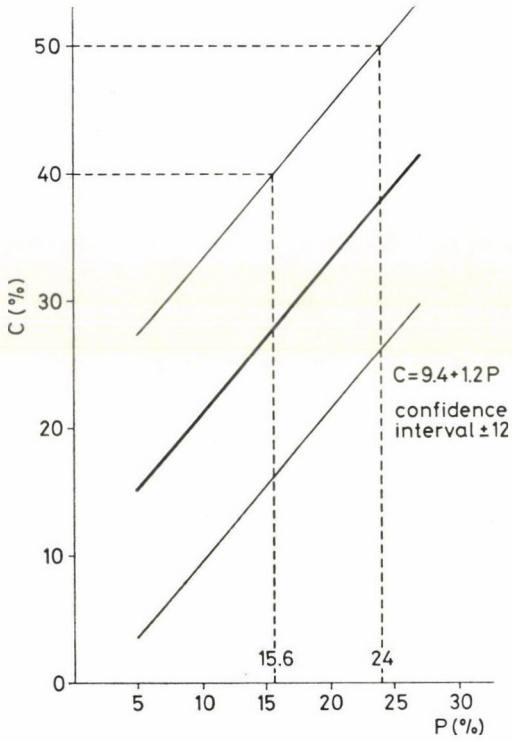

Fig. 2. General regression curve (heavy line) of Howard counts of tomato concentrate as a function of the Howard counts of the pulp, showing the confidence zone (1970–73)

fidence zone the value belonging to a given P value may vary. The width of the confidence zone is ± 12 Howard count units.

The Howard counts of pulps belonging to the specified Howard counts of the product were obtained by drawing a perpendicular from the point of intersection of co-ordinate C and the upper zone of confidence interval to co-ordinate P.

3. Conclusions

It can be seen in Fig. 1 that the frequency distribution histogramme of the Howard counts in the pulp differs somewhat from the normal distribution. The histogramme of the concentrate indicates the possibility of two, partly overlapping distributions. The first distribution resembles that of the pulp. The probable maximum value of the second distribution appears in the region of critical Howard counts (49–50).

An explanation may be that in this critical region the analysts take more unidentifiable particles to be hyphae than in higher or lower regions of lesser importance.

On comparing the β and s_{CP} values in Table 1, belonging to pastes of 28 % and 39 %, respectively, no substantial difference may be observed. Thus the Howard count in the paste is independent of the degree of concentration.

The standard deviation of Howard counts is $s_{CP} \simeq 6$. It follows from this that the variance is 36 which is composed of 3 error components: error inherent in the method, error characterizing the relation of pulp to paste and residual error. VAS and co-workers (1959) also reported that in the low Howard count region characteristic of the pulp the error of the method is 4, therefore the coefficient of variation amounts to 16. Thus 20 units remain for the two other components.

The regression coefficient is higher than 1 (1.2), the Howard count in the final product is somewhat higher than expected on the basis of that in the pulp.

The difference may be due to the fragmentation of hyphae into smaller units by agitation during evaporation, as well as to the method of counting. The Howard count of the paste is determined at a refractive index of 9 %, which is substantially higher than the 4–5 % in the pulp. Apart from this, overcautious counting may also account for the difference as is presumed on the basis of the histogramme of the paste in Fig. 1.

As can be seen from the general regression line in Fig. 2, to a paste of a Howard count of 40 belongs a Howard count of maximum 15.6 in the pulp. Taking into consideration the confidence interval, from a pulp of a Howard count of 15.6 a paste of Howard count 16–40 may be produced. To produce a final product of Howard count 50 a pulp of a Howard count of maximum 24 may be used. A pulp of this Howard count permits production of a paste of Howard count 26–50.

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DETERMINATION OF AVAILABLE METHIONINE AND LYSINE IN HEAT TREATED SOYBEAN SAMPLES

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The effect of heat treatment on available methionine and lysine was studied in soybean samples. Heat treatment was carried out in a *Brady* 400 type extruder at temperatures ranging from 127 to 154°C. Available methionine was measured according to DÉVÉNYI and co-workers (1974), available lysine was determined by the modified method of ROACH and co-workers (1967).

The availability of methionine and lysine was found to be 100% within experimental error up to 144°C. At 154°C methionine availability decreased to 69%. In accordance with the chemical estimations the biological value of the samples treated at 154°C significantly decreased, too (52 compared to 77 measured in the sample treated at 127°C).

Heat treatment, one of the most frequent steps in food processing, has a complex effect on protein quality. It may improve the nutritive value by destroying undesirable substances, but it can also decrease the biological value (HARRIS & VON LOESECKE, 1960). The latter phenomenon is ascribed to the destruction of some amino acids and to changes in their biological availability. Biological values depend highly on the availability of the limiting amino acids.

Earlier studies (CARPENTER, 1960; ROACH *et al.*, 1967) paid great attention to the changes of the available lysine content. The chemical availability of lysine shows close correlation with the feeding tests (*e.g.* in the case of cereals).

Methionine is the limiting amino acid in the proteins of legumes (ALTSCHUL, 1968). During heat processing it may be oxidized to methionine sulphoxide (MESO) and sulphone ($MESO_2$). It is generally accepted, that $MESO_2$ is unavailable in the nutritional sense, but with respect to MESO there is no uniformity of opinion (NJAA, 1962; SLUMP & SCHREUDER, 1973). "Total methionine content" can be determined according to the method of MOORE (1963), where $MESO_2$ is measured after oxidation with performic acid. This technique does not furnish any information about the relative quantities of MET, MESO and $MESO_2$ present originally in the sample. During the usual acid hydrolysis unoxidized available MET suffers further changes. In plant materials a destruction is observed, which highly depends on the accompanying substances (oxidative agents, antioxidants, carbohydrates, *etc.*) and on the conditions of hydrolysis. This destruction can be corrected by calculating

individual recovery ratios for each sample according to the method of DÉVÉNYI and co-workers (1974), thus available MET can be determined directly.

In the present work the chemical availability of methionine and lysine was determined in soybean samples exposed to different heat treatments. The data are compared to biological values obtained by N balance tests.

1. Materials and methods

1.1. Heat treatment

Soybeans (ISZ–8) of 14% moisture content were heat treated in a *Brady* 400 type extruder. Duration of the heat effect was 20 seconds, pressure ranging from 2.5 to 5 atm according to the temperature applied. The processing temperature was measured in the extruder cone.

1.2. Hydrolysis

Extruded soybean samples were powdered and homogenized. Hydrolysis was carried out in sealed test tubes in 2.0 ml of 6 *N* HCl at 105 °C for 48 hours, under nitrogen atmosphere. Hydrochloric acid was neutralized with 4.0 ml of neutralizing solution (10.5 g of NaOH dissolved in 100 ml of sodium citrate buffer, pH = 2.2) according to SPITZ (1973). Neutralization was omitted in the case of fluorodinitrobenzene (FDNB) treated samples in order to avoid the interference of DNP-derivatives.

1.3. Amino acid analysis

Analysis was carried out on a *Beckman Unichrome* analyser, except $MESO_2$ which was measured on a *Biocal* BC 200 analyser. The conditions of the analysis are shown in Table 1.

Table 1

Amino acid analyser programming procedures

	[1]Methionine	[2]Lysine	Methionine sulphone
Column	0.8×14 cm	0.8×14 cm	0.8×63 cm
Buffer flow rate	100 ml h^{-1}	100 ml h^{-1}	50 ml h^{-1}
Buffer 1 (pH = 3.28, Na^+ = 0.2 *M*)	0–25 min	0–25 min	0–70 min
Buffer 2 (pH = 4.25, Na^+ = 0.8 *M*)		25–40 min	–
0.2 *N* NaOH	25–27 min	40–45 min	70–100 min
Buffer 1	27–40 min	45–60 min	100–150 min

[1] DÉVÉNYI and co-workers (1974)
[2] DÉVÉNYI (1971)

1.4. Determination of the total methionine content

Samples of 20.0 mg were treated in test tubes with 0.5 ml of performic acid for 30 min at room temperature. Excess performic acid was destroyed with 0.15 ml of HBr (30%) according to MOORE (1963). During this time the test tube was cooled in order to prevent foaming. The sample was evaporated in vacuo, then hydrolysed and neutralized as described above. 0.3 ml of the solution corresponding to 1.0 mg of the sample was analysed, the amount of $MESO_2$ was determined. The total MET content measured after oxidation (MET^{ox}) can be expressed as follows:

$$MET^{ox}\ [\%(w/w)\ sample] = 0.82 \cdot MESO_2\ [\%(w/w)\ sample]$$

where 0.82 is the ratio of the molecular weights.

1.5. Determination of the available methionine content

For the determination of available MET the method of DÉVÉNYI and co-workers (1974) was followed. This method determines the hydrolytic destruction of MET by the addition of known amounts of MET. The recovery ratio is then calculated by the following formula:

$$\text{recovery ratio} = \frac{MET^x - MET^a}{MET^s}$$

where MET^s = MET measured in the sample (no added MET)
MET^x = MET measured in the sample in the presence of added MET
MET^a = MET added to the sample.

The calculation is shown by the following example:

Untreated soybeans

Sample No.	MET^a	MET^s	MET^x	$\frac{MET^x - MET^s}{MET^a}$
	(μmole per 4 mg of sample)			
I	–	0.11 0.11		
II	0.068		0.16	0.735
III	0.137		0.21	0.729
IV	0.205		0.25	0.683 (omitted from the average)
V	0.410		0.41	0.731
		Average recovery ratio:		0.731

$MET^c = 0.11/0.731$ [μmole per 4 mg sample] = 0.56 [%(w/w) sample].

For one determination five samples of 40.0 mg were prepared. MET^s was determined from one of them as described above. The other samples were used to determine the recovery ratio as follows: a MET solution was prepared dissolving 7.5 mg of MET in 10 ml of 6 *N* HCl. (The exact MET content was determined by analysis.) 0.1, 0.2, 0.3, 0.6 ml of MET-solution were added to the samples, than the content of the tubes was filled up to 2.0 ml with 6 *N* HCl. Hydrolysis and analysis were carried out as described above. The calculated available MET content (MET^c) was obtained by dividing MET^s by the average recovery ratio.

In the given concentration range recovery ratios were found to be equal within an estimated experimental error of about 5%. Thus MET^x plotted against MET^a showed a linear relation. This diagram was made for each available MET determination in order to check the data.

The availability of MET was calculated according to the following formula:

$$\text{methionine availability } \% = \frac{MET^x}{MET^{ox}} \cdot 100$$

1.6. Determination of available lysine

For the determination of available lysine a modified version of the method of ROACH and co-workers (1967) was used. It consists in measuring available lysine as the difference between the "total" value (LYS^t) obtained by acid hydrolysis, and the "residual" value (LYS^r) determined as free lysine present in the acid hydrolysate of the sample pretreated with fluorodinitrobenzene. Lysine availability can be expressed as follows:

$$\text{lysine availability } \% = \frac{LYS^t - LYS^r}{LYS^t} \cdot 100$$

The numerator is the available lysine content.

For the determination of the LYS^t values samples of 20.0 mg were hydrolyzed and analysed as described above. LYS^r values were measured in the following way: samples of 10.0 mg were soaked with 0.1 ml of $NaHCO_3$ solution (10% w/v in water) for 10 min, then 0.2 ml of FDNB solution (5% v/v in alcohol) was added. The mixture was shaken for 2 hours at room temperature, then the reaction was stopped with 0.05 ml cc HCl (30%). Alcohol was evaporated on a steam bath, then the samples were evaporated to dryness in vacuo. Hydrolysis was carried out in 8.0 ml of 6 *N* HCl. The hydrolysates were evaporated in vacuo at 60 °C over solid P_2O_5 and KOH. The dried residue was redissolved in 2.0 ml of deionized water; 0.6 ml of the solution corresponding to 3 mg of the sample was analysed as described above.

1.7. Biological evaluation

Evaluation of protein quality was carried out by N balance tests according to the method of BOCK and co-workers (1964) with groups of six albino rats.

Untreated soybeans and samples treated at 127 and 154 °C were tested for biological value (BV). The measured BV's are 47, 77 and 52, respectively.

2. Results

Available methionine and lysine contents are summarized in Table 2.

It is generally accepted that data obtained by amino acid analysis are reliable within a relative error of 3% (MOORE, 1972). The error of quantities derived from analysis data must surpass this limit. Thus the relative error

Table 2

Available methionine and lysine content of heat treated soybean samples

Temperature of heat treatment[3]	[1]Lysine			[2]Methionine		
	Total (%, w/w)	Available (%, w/w)	Availability (%)	Total (%, w/w)	Available (%, w/w)	Availability (%)
–	2.63	2.63	100	0.57	0.56	99
127	2.65	2.65	100	0.56	0.57	102
132	2.56	2.56	100	0.58	0.57	99
135	2.63	2.63	100	0.56	0.57	101
138	2.64	2.64	100	0.54	0.54	100
144	2.63	2.63	100	0.52	0.49	94
149	2.34	1.94	83	0.55	0.49	90
154	2.19	1.61	69	0.55	0.48	87

[1] Values are averages of triplicate determinations
[2] Values are averages of duplicate determinations
[3] For details of heat treatment see para. 1.1

of availability values may be estimated at 6% in first approach. In repeated assays on the same heat treated material we have found that availability values were reproducible within a relative error of 3–5%. The precision of the $MESO_2$ and lysine determination is discussed in the original papers. Both methods are reliable within a relative error of 3% and show quantitative recovery. So LYS^r values less than 3% LYS^t were neglected in our assays, in these cases lysine availability was taken as 100%.

The reproducibility of available methionine determination was checked in a series of tests, using untreated soybeans as test material. The data are summarized in Table 3.

The standard deviation of the MET^c values (2.1%) meets the requirements of amino acid analysis. The data presented in Table 3 confirmed our previous finding that recovery ratio and MET^s can vary due to some differences in the conditions of hydrolysis but this variance does not affect the calculated MET^c content.

Table 3

Variability of available methionine content measured in untreated soybeans

[2]Experiment	[1]MET^s	[1]MET^x	[1]MET^d	Recovery	[1]MET^c
A	.121	.160	.050	.780	.155
	.115	.193	.100	.780	.147
	.118	.237	.150	.793	.149
Average	.118			.784	.150
B	.130	.174	.052	.846	.153
	.127	.217	.104	.865	.147
	.125	.259	.156	.859	.146
Average	.127			.857	.149
C	.121	.167	.056	.821	.149
	.122	.212	.112	.803	.151
	.121	.257	.168	.810	.149
Average	.121			.811	.150

Available methionine content:

$MET^c = 0.150 \pm 0.001$ μmole per 40 mg sample
Standard deviation is 2.1% of the mean value

[1] Values expressed as μmole per 40 mg sample

[2] Separate experiments with freshly prepared reagent solutions. Values were measured in different tubes. Data in the same lines were obtained from tubes hydrolyzed and analysed at the same time

3. Conclusions

The results show that in the conditions of the applied extrusion technique heat treatment impairs methionine and lysine availability only above 149 °C. Up to this temperature both availability values were found to be 100% within the experimental error. At 152 °C methionine availability decreased to 87%, that of lysine decreased to 69%. At the same temperature of heat treatment the total lysine content showed a decrease of 17% as compared to untreated soybeans.

The biological value (BV) was found to be 48 in untreated soybeans. Heat treatment at 127 °C increased BV to 77. This phenomenon is explained by the destruction of the trypsin inhibitor content. The sample treated at 154 °C showed a BV of 52, indicating the detrimental effect of the excessive

heat treatment. (A more detailed discussion of the biological assays will be published elsewhere.)

The comparison of chemical and nutritional data suggests that these chemical methods offer valuable means for screening protein quality, especially when a large number of samples is to be tested. Chemical tests obviously do not replace biological evaluation, but, as it was shown in the present work, furnish sufficient information to reduce the number of the more expensive and time-consuming animal trials.

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THE SPOROSTATIC EFFECT OF CANNABIDIOLIC ACID

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A study was undertaken to clarify whether cannabidiolic acid could be made to enhance the microbiological effect of food preservation by heat treatment or irradiation.

Cannabidiolic acid was found to have a high inhibiting effect on the spores of *Bacillus cereus*. Its sporostatic effect is roughly equivalent to that of the antibiotics: nisin and tylosin.

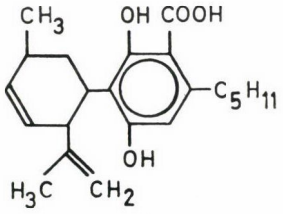
The aqueous solution of the phytoncide of 50 $\mu g\,ml^{-1}$ concentration (containing 2.5% alcohol) resisted, without a reduction of its activity, a heat treatment of 30 min at 110 °C. Its activity was reduced by only 15% upon treatment with 400 krad.

Hereafter the influence of cannabidiolic acid, added to the brine of canned peas at a concentration of 10 $\mu g\,ml^{-1}$, was studied on samples (a) exposed to treatment with 500 krad or (b) given a heat treatment equivalent to $F_0 = 4.8$. In these combination treatments cannabidiolic acid added at the above concentration proved to be ineffective. On investigating the cause of this phenomenon, cannabidiolic acid was found to react with protein of peas prior to irradiation or heat treatment, or in an early phase of treatment loosing thereby its microbiological effect. On the other hand, since cannabidiolic acid cannot react with proteins denatured by heat, it was found active in a sterilized nutrient medium containing denatured protein.

Usually, in the microbial flora of foods bacterial spores show the highest resistance to bactericidal effects, such as heat treatment or irradiation. Therefore in case of insufficient heat or radiation treatment bacterial spores are most likely to survive. However, if the development (germination or outgrowth) of the surviving spores can be inhibited by the application of some additive this combined treatment may highly increase the keeping quality of the product given a heat or radiation treatment at a significantly reduced level.

In this study we tried to find out whether cannabidiolic acid was suitable to reduce the dose requirement of radiation preservation.

Cannabidiolic acid was isolated from hemp by FERENCZY and co-workers (FERENCZY, 1956; FERENCZY *et al.*, 1958) and was identified as a phytoncide of inhibiting effect upon Gram positive bacteria. It was found ineffective against Gram negative bacteria and fungi. Its chemical composition (KREJČI *et al.*, 1958; SCHULTZ & HAFFNER, 1958, 1960) is as follows:



Because of the cheapness of its raw material (the residue of seed-hemp threshing) and its innocuity it was tested for use in pharmacology (KREJČI, 1958) and in food microbiology (GÁL & VAJDA, 1968). GÁL and his co-workers (1969) investigated certain factors affecting the activity of cannabidiolic acid and developed a thin-layer chromatography method for its detection.

In view of our aim we investigated

a) whether cannabidiolic acid acts against spores of *Bacillus cereus*, frequently occurring in low-acid foods,

b) which of the developmental stages of the spore forming bacterium is most susceptible to cannabidiolic acid.

The simplest procedure of combination treatment would be to add the cannabidiolic acid to the food to be preserved prior to heat or radiation treatment. The success of this procedure is dependent on the heat or radiation resistance of the cannabidiolic acid, whether or not its activity is reduced by the heat treatment or irradiation applied. Thus, the heat and radiation tolerance of the phytoncide was studied and some laboratory experiments were carried out to find out its applicability in pea canning.

1. Materials and methods

The material used in the experiments was a preparation produced at the PLANT PHYSIOLOGY DEPARTMENT, JÓZSEF ATTILA UNIVERSITY, Szeged. The preparation was the triethylamine salt of cannabidiolic acid (FERENCZY *et al.*, 1958; FERENCZY, 1968).

The preparation was difficult to dissolve in cold water, the solubility was below 0.1 g l^{-1}. However, it dissolved readily in alcohol at room temperature (*cca* 20 g l^{-1}). Therefore, the solutions used in the experiments were prepared by diluting appropriately the alcoholic stock solution with water. (No precipitation was observed during dilution.)

1.1. The sporostatic effect of cannabidiolic acid

To establish the sporostatic effect of cannabidiolic acid the turbidimetric method, developed in our laboratory for the measurement of nisin and tylosin lactate activity (FARKAS *et al.*, 1967), was used. The incubation and

measurements were performed in a *Biophotometer* (DE BONET-MAURY & JOUAN, Paris).

The heat-activated spores of a *Bacillus cereus* strain, taken from the Culture Collection of the CENTRAL FOOD RESEARCH INSTITUTE, served as test organisms. The effect of alcohol, originating from the alcoholic stock solution of cannabidiolic acid was examined separately and it was found that solutions containing not more than 0.5% alcohol show no sporostatic effect. Thus the sporostatic effect observed was due solely to the phytoncide.

1.2. Distribution of Bacillus cereus spores according to their resistance to cannabidiolic acid

The resistance of the *Bacillus cereus* spores was established by plate-counting on a universal nutrient medium* of varied cannabidiolic acid content with a dilution series prepared from a heat-activated suspension of 10^8 ml^{-1} spore concentration. (The appropriate amount of the phytoncide solution was added to the molten, sterile nutrient agar immediately before plating.) After a 96-hour incubation period at 35 °C the number of colonies formed was counted.

1.3. Heat and radiation resistance of cannabidiolic acid

The aqueous solution (containing 2.5% alcohol) of the phytoncide (50 μg ml^{-1}) was exposed to a 30 min heat treatment at 110 °C or to an X-ray treatment with 50 to 600 krad (0.5 to 6.0 kGy), respectively. Subsequently the residual activity was determined in appropriately diluted solutions.

1.4. Radiation sources used in the experiments

A MEDICOR *Stabil 250* type, X-ray apparatus for material testing, was used for the resistance experiments, with a dose rate of about 280 krad h^{-1} in the place of the sample. In the combined preservation experiments a panoramic ^{60}Co gamma source of about 1.3 kCi (48.1 TBq) activity was used. The dose rate was 25 krad h^{-1}.

The X-ray treatment was carried out at room temperature, the gamma radiation source was placed in a chamber of 8–10 °C.

* Composition of the nutrient: meat 4 g, sweet whey 200 ml, tenfold diluted yeast extract 100 ml, glucose 10 g, peptone 5 g, distilled water 700 ml, with 2% agar (pH = 7.2).

1.5. Combination treatments

Peas were placed in small jars and filled up with a brine containing 1.5% common salt, 4% granulated sugar, 10 μg ml^{-1} cannabidiolic acid. The net weight of peas per jar was about 7 g and the brine was about 8 ml. Samples prepared with brine without cannabidiolic acid served as control. The pH of the brine after mixing with peas was 6.5 in all samples.

Part of the samples was treated in an autoclave. In order to establish the sterilizing value, temperature changes were followed in one sample by heat penetration measurements.

Another part of the samples was exposed to 500 krad gamma radiation. Fifty-two samples each were heat- or radiation-treated. Irradiated samples were stored at 2–10 °C and the heat-treated samples at 25° C and at 55 °C, respectively.

2. Results and conclusions

2.1. Sporostatic effect of cannabidiolic acid

Similarly to sporostatic antibiotics, cannabidiolic acid was found to affect mostly the outgrowth of spores. Under the conditions of activity measurement (incubation at 30 °C in a nutrient medium of pH = 6.6), and with a spore concentration of 10^7 ml^{-1}, the inhibition of outgrowth appears to be a linear function of the phytoncide concentration. The phytoncide concentration causing 100% inhibition was found to be about 6 μg ml^{-1} (Fig. 1).

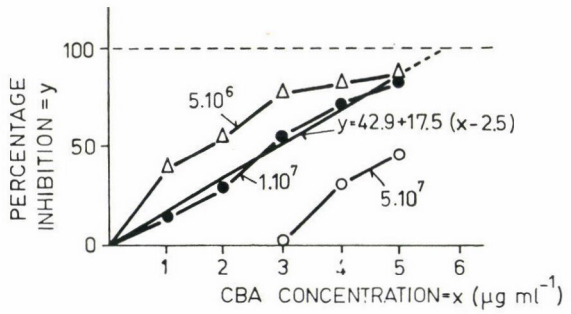


Fig. 1. The spore inhibiting effect of cannabidiolic acid (CBA) as a function of CBA concentration and cell density

In an earlier experiment (FARKAS *et al.*, 1967) the tylosin lactate concentration exerting complete inhibition was found to be 2.7 μg ml^{-1}, while that of Nisaplin 16.6 μg ml^{-1}. Thus, the sporostatic effect of cannabidiolic acid is of the same order of magnitude as that of these highly effective anti-

biotics as far as *Bacillus cereus* spores are concerned. As seen in Fig. 1, the effectiveness of the sporostatic preparation decreases with increasing spore concentration. Above spore concentrations of $5 \cdot 10^7$ ml^{-1}, however, germination and outgrowth of spores was not complete even in suspensions without additive.

The colony-forming capacity of *Bacillus cereus* spores as affected by cannabidiolic acid is shown in Table 1.

Table 1

The colony forming capacity of Bacillus cereus spores as affected by various cannabidiolic acid concentrations

Cannabidiolic acid in the nutrient medium (μg/ml)	Number of colony forming cells
0	$1.0 \cdot 10^8$
5	$1.5 \cdot 10^4$
10	$2.2 \cdot 10^1$

2.2. *Heat and radiation resistance of cannabidiolic acid*

The activity of phytoncide solutions was not reduced by heat treatment. The reduction of activity as caused by irradiation is shown in Fig. 2.

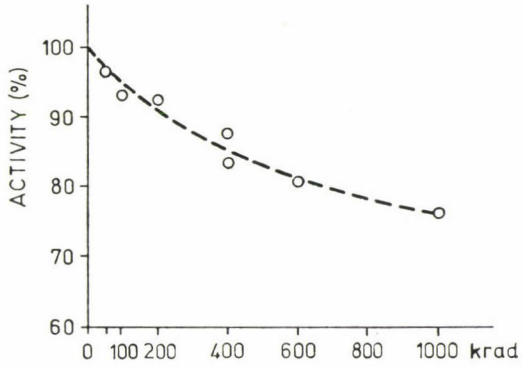


Fig. 2. The reduction of the activity in a cannabidiolic acid solution of 5 μg ml^{-1} concentration upon radiation treatment

As seen in Fig. 2 the activity of the 50 μg ml^{-1} solution was reduced only by about 15% by treatment with 400 krad. Thus the radiation resistance of the cannabidiolic acid exceeds that of tylosin lactate as well as that of nisin. It was shown in earlier experiments that the activity of tylosin

lactate was reduced by about 50%, that of nisin by about 75% upon treatment with 400 krad (FARKAS *et al.*, 1966). Radiation resistance is however influenced also by the concentration of the solution: in more dilute solutions the breakdown is more extensive. When a cannabidiolic acid solution of 20 μg ml^{-1} is radiation-treated with 400 krad the reduction of activity amounts to about 30%, that is twofold.

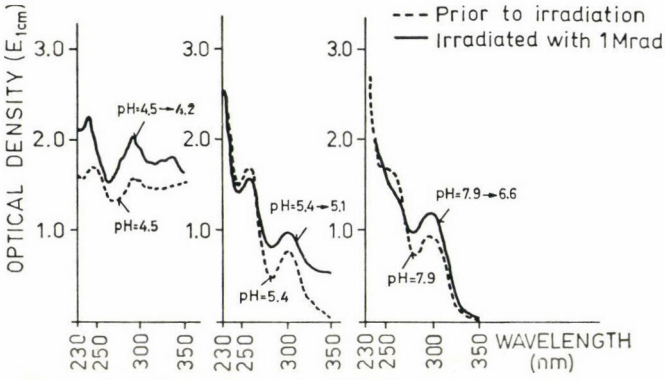


Fig. 3. The UV absorption spectrum of cannabidiolic acid solutions of different pH as affected by 1 Mrad

The UV spectrum of the phytoncide solution was also affected by irradiation. The absorption spectra of 100 μg ml^{-1} solutions of cannabidiolic acid set at various pH levels are shown in Fig. 3.

The diagram in the center of Fig. 3 illustrates the spectrum of solutions made with distilled water, containing 5% (v/v) alcohol. The diagram on the left side shows the solutions the pH of which had been set at 4.5, while those on the right side those set to 7.9, prior to irradiation. (The pH of the control solutions used in spectrophotometry was adjusted to the same level.) The pH of the phytoncide solutions was reduced every time by irradiation with 1 Mrad. Changes in the pH are also illustrated in Fig. 3.

2.3. Combination treatments

Two combined treatments were carried out. In the first experiment the phytoncide addition was combined with heat treatment and the sterilizing value applied was $F_0 = 4.8$. When the phytoncide was combined with irradiation the dose applied was 500 krad. In each case sufficient cannabidiolic acid stock solution was added to the brine to obtain 10 μg phytoncide per 1 ml brine.

Data on spoilage of the samples are given in Tables 2 and 3.

Table 2

Spoilage of heat-treated ($F_0 = 4.8$) peas

Storage temperature (°C)	Storage period (days)	Samples containing cannabidiolic acid			Samples without cannabidiolic acid		
		Spoilage percentage (%)	pH	microscopic picture	Spoilage percentage (%)	pH	microscopic picture
			of the spoiled samples			of the spoiled samples	
55	1	3.8	5.2	spore-forming bacilli	4.0	5.2	spore-forming bacilli
	2	23.0	5.0–5.3	spore-forming bacilli	28.0	5.0–5.2	spore-forming bacilli
	3	23.0	5.0–5.3	spore-forming bacilli	32.0	4.0–5.2	spore-forming bacilli
	10	23.0	5.0–5.3	spore-forming bacilli	36.0	4.0–5.2	spore-forming bacilli
	17	23.0	5.0–5.3	spore-forming bacilli	36.0	4.0–5.2	spore-forming bacilli
25	1	0	–	–	0	–	–
	2	0	–	–	0	–	–
	3	0	–	–	0	-	–
	10	0	–	–	4.0	5.7	spore-forming bacilli
	17	0	–	–	4.0	5.7	spore-forming bacilli

Table 3

Spoilage of pea samples irradiated with 500 krad and kept in refrigerated storage

Storage period (days)	Samples containing cannabidiolic acid		
	Spoilage percentage (%)	pH	microscopic picture
		of the spoiled samples	
10	18.9	3.2–4.0	mainly rods, some yeasts and cocci
17	58.2	3.2–6.4	mainly rods, some yeasts and cocci
68	69.8	3.2–6.4	mainly rods, some yeasts and cocci
	Samples without cannabidiolic acid		
10	16.0	3.2–4.0	mainly rods, some yeasts and cocci
17	52.3	3.0–6.4	mainly rods, some yeasts and cocci
68	60.0	3.0–6.4	mainly rods, some yeasts and cocci

As seen in Table 2, heat treatment at $F_0 = 4.8$ was not sufficient to kill thermophilic, spore forming, flat-sour microorganisms. Thus, both the samples containing and not containing cannabidiolic acid spoiled to a large extent during the two-week storage at 55 °C. The mesophilic bacteria causing spoilage

at 25 °C were more sensitive than the thermophilic microflora. The samples containing cannabidiolic acid and stored at 55 °C showed a spoilage of 23%, while those containing no phytoncide 36%. Because of the low number of samples the differences in spoilage percentages are not decisive, thus it cannot be definitely established whether or not the efficiency of heat treatment was substantially improved by the addition of cannabidiolic acid.

Table 3 shows that the efficiency of irradiation was not improved at all by the addition of cannabidiolic acid. It is of interest to note that, in the samples which were treated with 500 krad, stored under refrigeration and spoiled, not only spore forming bacteria, but yeasts and cocci were also present.

Already on the second day of storage, turbidimetric measurements failed to demonstrate the presence of any sporostatic effect in the samples given either heat or radiation treatments and containing cannabidiolic acid added prior to the treatments. Thus it was concluded that the phytoncide was inactivated by the combined effect of the substances present in the brine and heat or radiation treatment.

2.4. Elucidation of the causes of inefficiency of cannabidiolic acid in the preservation experiments

In pure solutions, cannabidiolic acid proved heat- and radiation-resistant and particularly efficient in the destruction of the spores of the mesophilic *Bacillus cereus*. Yet when applied in combination treatments it was ineffective. This phenomenon had to be investigated.

First the role played by the components of the brine was examined. The phytoncide* retained its activity in brines containing 4% sugar, and 1.5% common salt, when given a heat treatment equivalent to $F_0 = 3.3$, as long as it did not contact peas. Thus the components of the brine were not responsible for the inactivation of cannabidiolic acid. Consequently it seemed plausible that inactivation was due to substances released from the peas. However, when peas were boiled for 60 min in distilled water (1 : 1) and the extract was given a heat treatment of 30 min at 110 °C, in the resulting extract, cannabidiolic acid exerted the same sporostatic effect on *Bacillus cereus* spores as in the universal nutrient medium (Fig. 4).

It was presumed that the substances inactivating cannabidiolic acid in bottled peas were inactivated during the preparation of the extract or during its heat treatment. Therefore it seemed probable that, in the preservation experiments, cannabidiolic acid reacted with heat-sensitive substances, *e.g.* proteins even prior to heat or radiation treatment, or in the first phase of the

* Phytoncide concentration in the brine: 50 $\mu g\ ml^{-1}$.

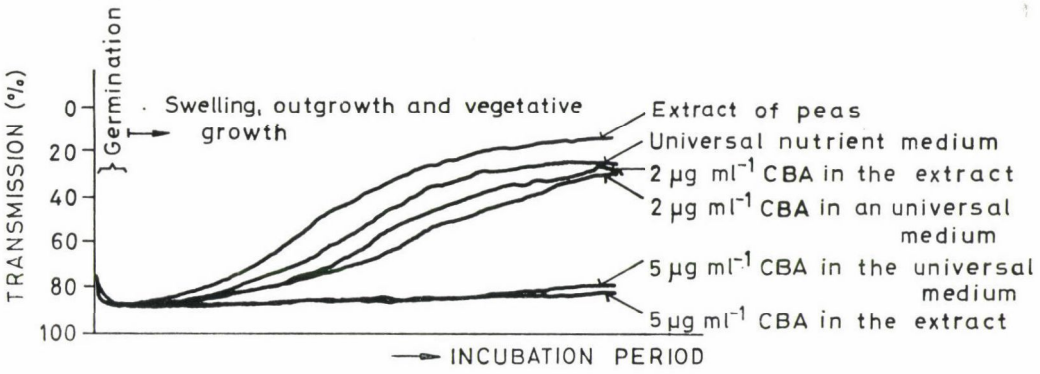


Fig. 4. Sporostatic effect of cannabidiolic acid in heat-sterilized pea extract and in universal nutrient medium. Change of the light transmission in samples inoculated with *Bacillus cereus* spores (Record of the measurement in the *Biophotometer*)

treatment, loosing at the same time its microbicidal effect. On the other hand, it apparently did not react with the proteins *denatured* by heat treatment. Thus, when added to solutions containing denatured proteins, it proved effective. This hypothesis was proven in further studies. In these experiments the universal nutrient medium was mixed with bovine-serum albumin. If the albumin solution was heat-treated prior to mixing with the nutrient solution, or together with the medium, its inactivating effect on the phytoncide was substantially lowered (Table 4).

Table 4

Influence of bovine-serum albumin on the sporostatic effect of cannabidiolic acid at a concentration of 5 µg ml⁻¹
(Spore concentration $1 \cdot 10^7$/ml)

Serum albumin concentration in the medium*		Relative sporostatic activity**
Albumin not heat-treated, (%)	Albumin heat-treated,*** (%)	
0	0	1.00
0.005	–	1.02
0.01	–	0.30
0.05	–	0.057
–	0.1	1.00
–	0.5	0.25

* Medium: universal nutrient broth

** Relative sporostatic activity: quotient of the inhibition values as measured in the albumin-containing solution and the medium not containing albumin

*** The nutrient medium containing the albumin was given heat treatment in an autoclave for 30 min at 110°C

Thus it was proven that the failure of cannabidiolic acid to exert phytoncidal effect in bottled peas, was due to inactivation caused by a reaction with the native protein content of the peas to be preserved. This inactivation did not occur in the experiments to show the sporostatic effect of cannabidiolic acid, because the latter did not react with the denatured proteins in the medium previously sterilized in a retort.

These experiments confirm the findings of GÁL and co-workers (1969) inasmuch as the phytoncide proved heat-stable in nearly neutral solution. Its radiation resistance is also fairly high. Our observations on the loss of activity in media containing native protein support those of KREJČI (1958) and FERENCZY (1968), who found blood and milk, resp., to inactivate cannabidiolic acid. This fact reduces the applicability of cannabidiolic acid in the canning industry, it does not seem suitable for use in the preservation of raw foods containing native protein of plant or animal origin, to improve the efficiency of heat treatment or particularly of irradiation.

It requires further investigations, however to, clarify whether the compound is effective in reducing the final heat or radiation treatment requirements when added to previously heat-treated (blanched) foods. The degree of heat treatment (protein denaturation) above which no inactivation of cannabidiolic acid occurs should also be defined. It would be of interest to find out how cannabidiolic acid could be chemically modified to reduce its susceptibility to react with proteins. However, presumably this would reduce its antimicrobial effect as well.

*

Thanks are due to Dr. LAJOS FERENCZY, Reader (MICROBIOLOGY DEPARTMENT, JÓZSEF ATTILA UNIVERSITY OF SCIENCES, Szeged) for putting the crystallized phytoncide preparation at our disposal.

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A METHOD FOR DETERMINING POLYPHENOL OXIDASE ACTIVITY IN FRUITS AND VEGETABLES APPLYING A NATURAL SUBSTRATE

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An assay method was developed to determine polyphenol oxidase (*o*-diphenol oxygen oxidoreductase, E. C. 1.10.3.1, hereafter referred to as PPO) activity in fruit and vegetable homogenates on chlorogenic acid, a substrate occurring in these products. Potatoes of the variety *Gül baba* were applied to establish the conditions of measurement. The method is based on the measurement of the changes in absorbance of the coloured products formed upon interaction of the enzyme with chlorogenic acid. The relationship between optical density (OD) and reaction time was linear during the first 5 to 10 min. Reaction rate varied with substrate concentration according to a maximum curve. Maximum reaction rate was reached at 3.4 10^{-2} *M* chlorogenic acid content in the reaction mixture and this concentration was selected for activity determinations. The relationship between activity and reaction rate was found to be approximately linear in the concentration range of 2.0 to 3.2% (w/v) of potato homogenate in the reaction mixture.

PPO activity is expressed in terms of initial reaction rate as indicated by the regression coefficient in the equation describing the linear section of the OD *vs.* reaction time plot. The enzyme is considered to be of unit activity if it brings about a change in OD modulus of $1 \cdot 10^{-4}$ per min. Activities are expressed in terms of enzyme concentration, *i.e.*, as related to 1 g of product.

PPO concentrations of potatoes of the variety *Gül baba* as determined at pH 6.2 and 30 °C were found to be 1.50 and 1.21 kU g^{-1} resp., in different lots. Enzyme concentrations in kU g^{-1} of some fruits as assessed at pH 5.4 and 30 °C were as follows: peaches, variety *Elberta:* 0.28, variety *Ford:* 0.70 and 0.81, resp., in various lots; apples, different varieties: from 0.56 to 2.91; apricots (variety not specified): 2.16 and pears, variety *Alexander:* 4.42.

Accuracy and reproducibility of the method proved satisfactory: standard deviations of PPO activities (regression coefficients) calculated from 6 to 12 parallel determinations were, in most cases, below 5%.

The method was found suitable to study the action of enzyme inhibitors and to establish the pH-dependence of activity in homogenates of various products.

In addition to an activity assay procedure based on pyrogallol oxidation as developed for studying enzymic browning of potatoes (VÁMOS-VIGYÁZÓ *et al.*, 1973c; VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1974), a method applying a natural substrate seemed necessary to determine PPO concentration in apples. In agreement with the literature, the above synthetic substrate, more or less suitable to assess the activity of the enzyme in stone fruits (LUH & PHITHAKPOL, 1972) or potatoes (SCHWERDTFEGER, 1970), was not found to be readily oxidized by the PPO of apples (PONTING & JOSLYN, 1948; HAREL *et al.*, 1964; VÁMOS-VIGYÁZÓ *et al.*, 1972).

From the numerous substrates of the enzyme (VÁMOS-VIGYÁZÓ & BALÁZS-SPRINCZ, 1970) occurring in fruits and vegetables chlorogenic acid was selected, because

– in a number of horticultural products it is present in relatively high concentrations (TÄUFEL & VOIGT, 1963; MONTIES, 1966a; MACHEIX, 1967, 1968; HERRMANN, 1973) and, therefore, was assumed to be readily oxidized by the PPO present in their tissues,

– it is considered, by some authors, the main factor in enzymic browning (HULME, 1958) which fact might be connected to its higher solubility (MONTIES, 1966b) as compared to the rest of oxidizable phenolic compounds in fruits, and

– it ranges among the best substrates for the enzyme in pome fruits (HAREL *et al.*, 1964).

The scope of the present work was to determine the best conditions for spectrophotometric activity measurement, *i.e.* to establish optimum composition of the reaction mixture (the respective concentrations of the products) as well as reaction duration and the appropriate pH.

The method developed was applied to determine PPO concentration and establish the pH dependence of activity in various horticultural products as well as to preliminary investigations of enzyme inhibition.

1. Materials and methods

1.1. Materials

1.1.1. Potatoes and fruits. The activity assay method was developed applying potatoes of the variety *Gül baba* as enzyme source.

Enzyme concentration has been assessed, in addition, in apples of the varieties *Jonathan*, *Jonadel*, *Jonared*, *Golden delicious*, *Starking* and *Staymared* as well as in pears of the variety *Alexander*, in peaches of the varieties *Ford* and *Elberta* and in apricots (variety not specified).

The pH-dependence of activity was established for potatoes and peaches and inhibition experiments were carried out with apples of the variety *Jonathan*.

With the exception of the apples all the products were purchased from primary producers at Budapest markets. The apples were grown in an orchard in North-Eastern Hungary (Újfehértó).

Potatoes and fruits have both been stored at +4 °C.

1.1.2. The substrate. Chlorogenic acid (grade *purum*) was supplied by FLUKA (Mw: 354.3, Mp.: 208–210 °C).

1.1.3. Buffer solutions. The substrate was dissolved for all the measurements in pH 7.2, 0.2 *M* Na_2HPO_4–NaH_2PO_4 buffer (72.0 ml 0.2 *M* Na_2HPO_4 + + 28.0 ml 0.2 *M* NaH_2PO_4) (DAWSON *et al.*, 1959).

Buffer solutions used to prepare potato and fruit homogenates, resp., for obtaining reaction mixtures of different pH values are listed in Tables 1 and 2.

The preparation of reaction mixtures of various pH values with chlorogenic acid as substrate involved some difficulties. One of them consisted of the fact that the homogenates had to be prepared with buffers of pH values not higher than 4.0 to prevent enzymic browning during comminution (MACHEIX,

Table 1

Buffer solutions used to prepare potato homogenates

pH of reaction mixture	pH of buffer	Composition of buffer*			
		Citric acid solution		Trisodium citrate solution	
		Concentration (*M*)	Amount (ml)	Concentration (*M*)	Amount (ml)
3.8	2.2	0.10	20.0	–	–
5.1	3.0	0.10	18.6	0.10	1.4
5.7	4.0	0.10	13.1	0.10	6.9
5.9	4.0	0.08	13.1	0.08	6.9
6.2	4.0	0.06	13.1	0.06	6.9
6.5	4.0	0.02	13.1	0.02	6.9

* After DAWSON and co-workers (1969)

Table 2

Buffer solutions used to prepare fruit homogenates

pH of reaction mixture	pH of buffer	Composition of buffer*			
		Citric acid solution		Trisodium citrate solution	
		Concentration (*M*)	Amount (ml)	Concentration (*M*)	Amount (ml)
3.8	2.2	0.10	20.0	–	–
4.3–4.4**	3.0	0.01	20.0	–	–
5.4	4.0	0.10	13.1	0.10	6.9
6.0	4.0	0.05	13.1	0.05	6.9
6.4	4.0	0.05	13.1	0.05	6.9

* After DAWSON and co-workers (1959)

** For peaches of pH 3.6–3.8 (variety *Ford*) the resulting pH of the reaction mixture was 4.3, for those of pH 4.0–4.2 (variety *Elberta*) it was 4.4

1970; VÁMOS-VIGYÁZÓ *et al.*, 1973c). On the other hand, dissolving of powdered chlorogenic acid had to be achieved, for reasons of solubility, in a buffer of a pH not below 7.2. It must be borne in mind that – with mixtures of the usual buffer capacities – the acidic character of the substrate decreases the pH of the buffer (*i.e.*, a NaH_2PO_4–Na_2HPO_4 buffer solution of pH 7.2 yields a 2% chlorogenic acid solution of pH 6.8). Another difficulty presents itself in the fact that the colour of the chlorogenic acid solution is pH-dependent. The light yellow colour of the solution prepared with the pH 7.2 buffer (see para. 1.1.3) is more or less stable for 1 or 2 hours, while, *e.g.* glycine–NaOH buffers in the pH range of 8 to 10 (DAWSON *et al.*, 1959) undergo rapid changes in air, probably due to auto-oxidation (INGRAHAM & CORSE, 1951).

Therefore it is expedient to adjust the pH values of the reaction mixtures for investigating the pH-dependence of PPO activity by altering the buffer capacities as can be seen in Tables 1 and 2. The tables show equally that the lower pH of the fruits investigated (3.6–4.4) also affected the resulting pH of the reaction mixtures as compared to that of the potatoes (6.2) and had to be taken into account in selecting the buffers.

1.1.4. Inhibitors. Ascorbic acid (REANAL, Budapest) was used in the inhibition experiments.

1.2. Methods

1.2.1. The principle of the activity assay method. From the various methods published in the literature colorimetric determinations seem most suited to assess PPO activity in homogenates of fruits or vegetables (WHITAKER, 1972; VÁMOS-VIGYÁZÓ *et al.*, 1973c). The underlying principle of the method is the spectrophotometric measurement of the initial rate of discolouration as induced by enzyme action in mixtures containing homogenates of the products and substrate.

1.2.2. Selection of the appropriate concentrations of enzyme and substrate and preparation of the reaction mixtures. In order to establish the concentrations of substrate and enzyme, resp., best suited for measurement, chlorogenic acid solutions of 1.0, 2.0, 3.0 and 4.0% (w/v) were prepared with the pH 7.2, 0.2 *M* Na_2HPO_4–NaH_2PO_4 buffer as well as a 10% (w/v) stock homogenate of potatoes with the pH 4.0, 0.06 *M* citric acid–sodium citrate buffer (Table 1). The latter was used undiluted as well as diluted to 8, 6 and 5% (w/v) with the same buffer.

The reaction mixtures were prepared to contain 6 ml chlorogenic acid solution and 4 ml homogenate each, while the blanks contained 6 ml chlorogenic acid solution and 4 ml buffer, pH 4.0, each.

This volume ratio was maintained in all the experiments (including those carried out with products other than potatoes).

Thus, the actual concentrations of enzyme and substrate in the reaction mixtures resulted to be as follows:

Concentration of the enzyme-containing homogenate in the reaction mixture	Concentration of the substrate in the reaction mixture	
(%, w/v)	(%, w/v)	10^{-2} *M*
2.0	0.6	1.7
2.4	1.2	3.4
3.2	1.8	5.1
4.0	2.4	6.8

1.2.3. Determination of PPO activity. 6-ml portions of chlorogenic acid solution were pipetted into 50 ml conical flasks and preheated to 30 °C on a water bath *Vibrotherm* (LABOR MIM, Budapest). After the substrate had reached this temperature, 4 ml each of the enzyme homogenate and, for blanks, 4 ml each of the pH 4.0 buffer, both preheated to 30 °C, were added. The combined reaction components were then incubated under shaking for various intervals up to 25 min.

At the end of each reaction period, *i.e.*, right on combining the components ("0 min") and then every five minutes OD-s were read at 420 nm in a spectrophotometer *MOM 203* (MOM, Budapest) against blanks incubated for identical intervals. Reaction mixtures were filtered through folded filters prior to taking OD readings.

In every series of measurements 3 parallel reaction mixtures were read against the same blank, at each interval. The experiments were repeated 2 to 4 times with different homogenates of the same product, thus activities were calculated from 6 to 12 parallel determinations. pH values of the blanks were checked in every measurement and were found to be identical with those of the respective reaction mixtures.

1.2.4. Investigations into the pH dependence of PPO activity. The buffers ensuring the appropriate pH values for activity measurement were prepared according to para. 1.1.3. The ratio of the reaction components and the experimental conditions were identical with those described in the preceding paragraphs.

1.2.5. Applying the assay method for determining PPO concentration in various horticultural products. Owing to considerable differences in PPO concentrations of the products investigated the homogenate content of the reaction mixtures had to be adjusted accordingly and resulted to be 1.0% (w/v) for pears, 3.2 to 4.0% (w/v) for potatoes and 4.0% for the rest of the products.

1.2.6. Measurement of enzyme inhibition. Inhibition of enzyme activity was measured on apples of the variety *Jonathan*. Since PPO activity of this product was found to be negligible near its own pH (around 4.0), inhibition was studied in reaction mixtures of pH 5.4.

Ascorbic acid was applied as inhibitor and added to the diced apples in concentrations of 1.0, 0.5 and 0.1% (w/v) as related to fruit weight, prior to buffer addition and further comminution. Homogenates of ascorbic acid treated and of untreated apples, resp., were prepared from 10 g each of the same fruits.

Activity measurements were carried out as in para. 1.2.3.

1.2.7. Biometrical evaluation of the results. Activities were calculated from individual OD data by linear regression. Standard deviations of the regression coefficients were determined (SVÁB, 1967).

2. Results

2.1. Relationship between OD and reaction time

The relationship between OD and reaction time (t, min) as studied in reaction mixtures containing 2.0, 2.4, 3.2 and 4.0% (w/v), resp., of potato homogenate is shown in Fig. 1.

With the exception of the homogenate concentration (C_H) of 2.0% (w/v) the plot was linear only for the first 5 or 10 min of the reaction. The OD values as obtained at "0" time differed from those of the respective blanks, the constants of the regression equations were $\neq 0$ and increased with C_H. This is attributed to the fact that – owing to manipulation – OD readings at "0" reaction time were carried out 3 min after combining the components of the reaction mixtures, when a certain discolouration had already occurred. The scattering of the "0"-time values is mainly due to the inhomogeneity of the samples (VÁMOS-VIGYÁZÓ *et al.*, 1973c). The substrate (*o*-dioxy phenol) content of the products investigated so far was found to be equivalent to 10–200 mg% chlorogenic acid (VÁMOS-VIGYÁZÓ *et al.*, 1973b; 1975). This corresponds, in the reaction mixture, to values lower by 2 to 4 orders of magnitude as compared to the concentration of the added substrate. Therefore the substrate content of the product was not taken into account in developing the activity assay method.

2.2. Relationship between reaction rate and substrate concentration

The relationship between reaction rate (v, Δ OD · min^{-1}) and substrate concentration (C_S%, w/v) was established with reaction mixtures containing 3.2% (w/v) potato homogenate and 0.6, 1.2, 1.8 and 2.4% (w/v) chlorogenic acid, resp. The results are shown in Fig. 2.

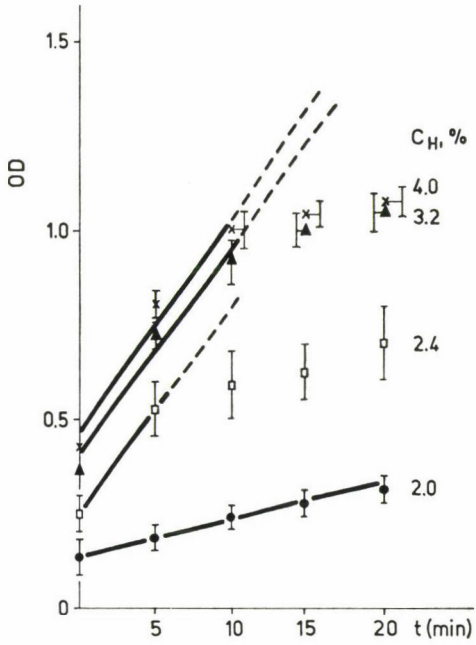

Fig. 1. Changes in optical density (OD) of reaction mixtures of various PPO contents with reaction time. – Potato concentrations (C_H) in the reaction mixtures: 4.0% (w/v), 3.2% (w/v), 2.4% (w/v) and 2.0% (w/v). Regression equations for the linear sections of the curves in above order of C_H:

OD = 0.435 + 0.060 t; r = 0.976; r^2 = 0.953; n = 18;
OD = 0.393 + 0.055 t; r = 0.949; r^2 = 0.900; n = 18;
OD = 0.258 + 0.054 t; (from data of 2 points of measurements);
OD = 0.128 + 0.010 t; r = 0.895; r^2 = 0.801; n = 45.

Regression equations were calculated from 6 to 12 individual data of OD at each point of measurement.

pH = 6.2; T = 30 °C; C_S = 1.2% (w/v) chlorogenic acid.

Vertical bars indicate standard deviations (2s). Experimental conditions as in Table 3

Reaction rate was found to vary with substrate concentration according to a maximum curve. Maximum reaction rate was obtained at a substrate concentration of 1.2% (w/v) which corresponds to 6 ml 2% (w/v) chlorogenic acid solution in 10 ml of reaction mixture (para. 1.2.3). This was adopted for further investigations, since higher substrate concentrations inhibit the enzyme.

2.3. Relationship between reaction rate and concentration of the enzyme-containing homogenate

The plot of reaction rate *vs.* enzyme concentration was found to fit a sigmoidal curve (Fig. 3) (Sváb, 1973).

The correlations as obtained with two lots of potatoes (A = autumn and B = early sample) proved significant at probability levels of 95 and 90%, resp. The parts around the inflexion points of the calculated curves can be

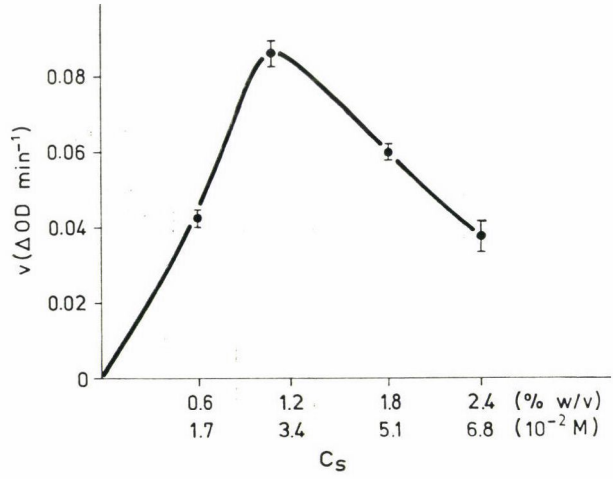

Fig. 2. Relationship between reaction rate and substrate concentration. – Source of enzyme: homogenate of potatoes, variety *Gül baba* (3.2%, w/v in the reaction mixture); v = reaction rate (Δ OD · min^{-1}); C_S = concentration of chlorogenic acid; pH = 6.2; T = 30 °C. Experimental conditions as in Table 3

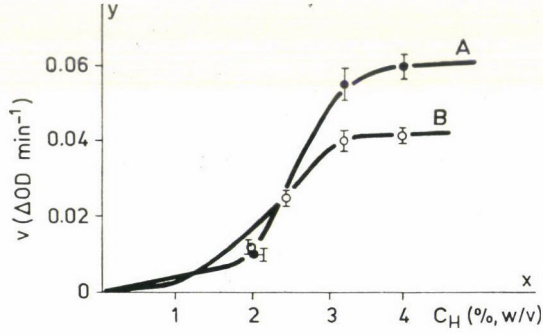

Fig. 3. Relationship between reaction rate and enzyme (potato homogenate) concentration. – v = reaction rate (Δ OD · min^{-1}); C_H = potato concentration in the reaction mixture (%, w/v); pH = 6.2; T = 30 °C; C_S = 1.2% (w/v) chlorogenic acid. Vertical bars indicate standard deviations (2s) calculated from 6 parallel determinations of v (regression coefficients). – Experimental conditions as in Table 3. Regression equation of the sigmoidal curves as obtained with two lots of potatoes (A = autumn and B = early sample):

$$\text{A:} \quad y = 0.061\left(\frac{1}{1 + 0.2548 \cdot 0.05623^{x-3}}\right);$$

$$r = 0.9971^{*} \quad (DF = 1)$$

$$\text{B:} \quad y = 0.044\left(\frac{1}{1 + 0.1720 \cdot 0.08856^{x-3}}\right);$$

$$r = 0.9032^{**} \quad (DF = 2)$$

(r = correlation coefficient; DF = degree of freedom; * and ** = correlations significant at the probability levels of 90 and 95%, resp.)

described, in the range of 2 to 3.2% (w/v) homogenate concentration, by linear equations (for lot A: $y = -0.062 + 0.036\ x$; $r = 0.9999^{***}$, $DF = 2$; for lot B: $y = -0.026 + 0.021\ x$; $r = 0.9958^{**}$, $DF = 2$; for the meaning of x, y, r and DF see Fig. 3; *** and ** indicate correlations significant at the probability levels of 99.9 and 99%, resp.). Thus, in the range of homogenate concentrations from 2 to 3.2% (w/v) the relationship between reaction rate and enzyme concentration may be considered linear.

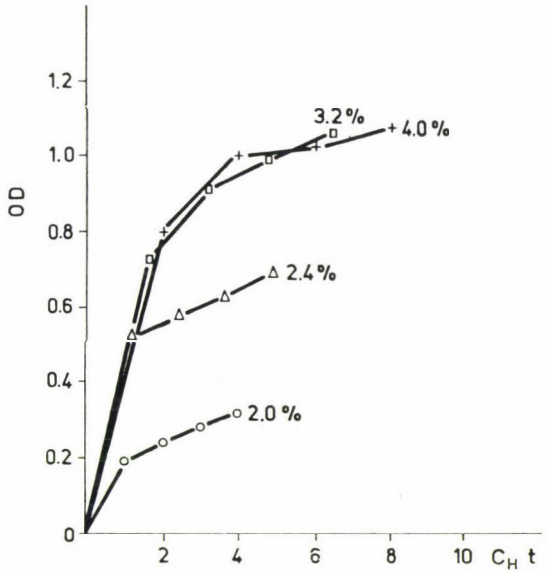

Fig. 4. Relationship between optical density (OD) and the product of enzyme (potato homogenate) concentration and reaction time (SELWYN, 1965). – C_H = potato concentration in the reaction mixture; pH = 6.2; T = 30 °C; C_S = 1.2% (w/v) chlorogenic acid. – Experimental conditions as in Table 3

2.4. Investigations into substrate saturation and enzyme inactivation during the reaction

Since PPO is reported to be inactivated during the reaction (WHITAKER, 1972) and higher substrate concentrations were found to inhibit the enzyme (Fig. 2) it seemed necessary to investigate the conditions selected for measurement from this aspect. A simple method of simultaneously checking substrate saturation and inactivation as suggested by SELWYN (1965) consists in plotting OD against the product of enzyme concentration (in the given case the concentration of the homogenate) and reaction time. If the criteria of substrate saturation are fulfilled and no inactivation occurs during the reaction, the OD values fit the same curve.

As can be seen in Fig. 4, OD values as obtained with homogenate concentrations of 3.2 and 4.0% (w/v), resp., yield a curve in common.

2.5. Unit of enzyme activity

PPO activity is characterized by initial reaction rate, *i.e.*, by the changes in OD per min as calculated from the linear sections of the OD *vs.* reaction time plots (Fig. 1). Enzyme activity is indicated by the slope of the regression curve, *i.e.*, the coefficient of the independent variable in the regression equation.

The enzyme is considered to be of unit activity, if it brings about a change in OD modulus of $1 \cdot 10^{-4}$ per min. To facilitate comparison, enzyme activities are expressed in kU (1 kU = 1000 U) related to 1 g of product (wet weight) and referred to as enzyme concentration.

Variation coefficients of enzyme activity values (regression coefficients) were, in most cases, below 5%.

2.6. Utilization of the assay method to study PPO concentration of various horticultural products

2.6.1. PPO concentrations of horticultural products. PPO concentrations of some horticultural products as listed in para. 1.1.1 are given in Table 3.

Considerable differences in PPO concentration could be observed between varieties of the same fruit: the highest value as found for apples (*Staymared*) was more than 5 times that of the lowest one (*Jonathan*, 1972). The difference in enzyme concentration of the *Jonathan* lots of two different years was nearly 70%. Pears had the highest PPO content of the pome fruits and, at the same time, of all the products included in this study. Of the stone fruits investigated the enzyme concentration of apricots was 2.6–7.5 times as high as that of the two varieties of peaches. The latter two differed considerably from each other with respect to enzyme concentration: those of the variety *Elberta* had the lowest PPO contents of all the products assessed, while in the two lots of the variety *Ford* the values were 2.6–2.8 times higher. The difference in enzyme concentration of the two lots of potatoes *Gül baba* from different years was not too marked.

2.6.2. pH dependence of PPO activity in the homogenates of some products. PPO activity was studied in potato and peach homogenates in the pH range of 3.8 to 6.3 (pH values of the products were as follows: peaches, variety *Ford*, 3.6–3.8; variety *Elberta*, 4.0–4.2; potatoes, variety *Gül baba*, 6.2). The results are summarized in Fig. 5.

At pH 3.8 activity was below detectability in both the potato and peach homogenates. With increasing pH activity of both varieties increased, but in the whole range investigated the pH – activity curve of the peaches *Elberta* remained under that of the peaches *Ford*.

Table 3

Polyphenol oxidase (PPO) concentration of some fruits and vegetables

Name of product	Concentration of homogenate in the reaction mixture (%, w/v)	PPO-concentration*	
		kU g^{-1}**	s***
Apples			
Jonathan, 1972	4.0	0.56	0.009
Jonathan, 1974	4.0	0.94	0.051
Jonadel	4.0	1.30	0.077
Golden delicious	4.0	1.71	0.089
Jonared	4.0	2.44	0.112
Starking	4.0	2.46	0.126
Staymared	4.0	2.91	0.169
Pears			
Alexander	1.0	4.42	0.096
Peaches			
Elberta	4.0	0.28	0.003
Ford, lot I	4.0	0.70	0.031
Ford, lot II	4.0	0.81	0.085
Apricots			
Variety not specified	4.0	2.16	0.057
Potatoes			
Gül baba, 1973	3.2	1.50	0.081
Gül baba, 1975	4.0	1.21	0.065

* PPO concentration of products was assessed at 30°C in reaction mixtures containing $3.4 \cdot 10^{-2}$ *M* chlorogenic acid substrate and incubated for 0 to 25 min. pH of the reaction mixture was 6.2 for potatoes and 5.4 for the rest of the products. Reaction mixtures contained 6 ml 2% (w/v) chlorogenic acid (pH 7.2) and 4 ml pH 4.0 fruit or vegetable homogenate. Blanks contained pH 4.0 buffer instead of homogenate. OD values of the reaction mixtures were read at 5 min intervals at 420 nm against blanks incubated for identical periods. Initial reaction rates were calculated from the linear sections of the OD *vs.* reaction time plots

** The enzyme was considered to be of unit activity, if it brought about a change in OD modulus of $1 \cdot 10^{-4}$ per min. Enzyme activity was expressed in kU (1 kU = 1000 U), related to 1 g of product (wet weight) and referred to as enzyme concentration

*** Standard deviations of enzyme concentrations were calculated from the respective activities (regression coefficients) considering individual OD values as obtained from 6 to 12 parallel determinations

The pH dependence of potato PPO activity was markedly different. It remained near 0 between pH 3.8 and 5.7, then increased abruptly up to pH 6.2 and did not vary significantly with further increase in pH up to 6.5.

PPO activity of the two peach varieties investigated was low or even

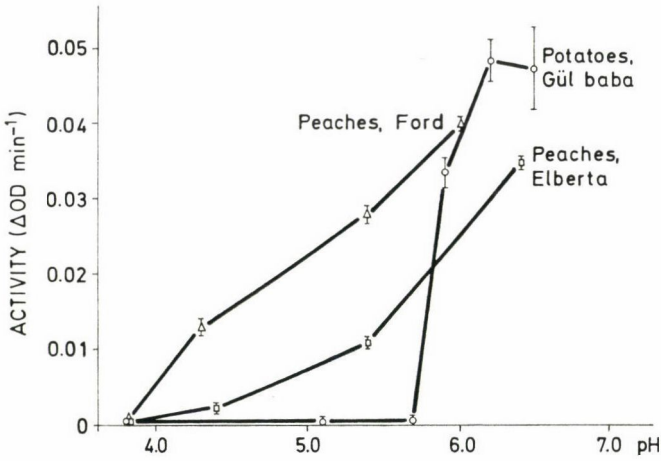

Fig. 5. Polyphenol oxidase (PPO) activity of horticultural products as a function of pH. – C_S = 1.2% (w/v) chlorogenic acid; T = 30 °C. Concentration of peaches and potatoes, resp., in the reaction mixture = 4.0% (w/v). Vertical bars indicate standard deviations, (2s) calculated from 6 to 12 parallel determinations of regression coefficients. – Experimental conditions as in Table 3

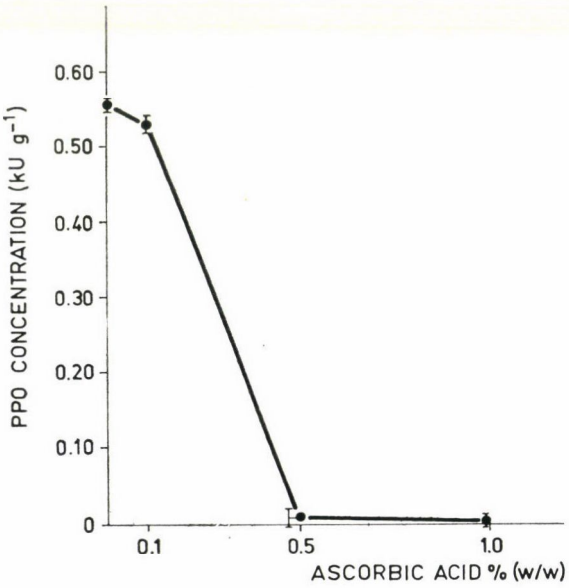

Fig. 6. Inhibition of PPO activity of apples of the variety *Jonathan* with ascorbic acid. – pH = 5.4; T = 30 °C; C_S = 1.2% (w/v) chlorogenic acid. Vertical bars indicate standard deviations (2s) calculated from 6 to 12 parallel determinations of regression coefficients. Apple concentration in the reaction mixture: 4.0%. Ascorbic acid has been added prior to preparing the homogenate. – Experimental conditions as in Table 3

below detectability around the pH of the products (3.8–4.4), while potatoes had a considerable activity at their own pH (6.2).

2.6.3. Investigations into enzyme inhibition. The relationship between PPO activity of apples of the variety *Jonathan* and the concentration of added ascorbic acid is shown in Fig. 6.

Complete inhibition of activity could be achieved with 1.0% (w/w) of added ascorbic acid as related to fruit wet weight, but even half this concentration proved sufficient to bring about a reduction in activity of 97% (the activity of 0.56 kU g^{-1} of untreated apple homogenate was reduced to 0.01 kU g^{-1}). However, a further decrease in inhibitor concentration (to 0.1% w/w) resulted in a poor effect: reduction of activity was only 4.5%.

The pH of the apple homogenate or the reaction mixture (4.0 and 5.4, resp.) was not altered by the addition of ascorbic acid in the concentrations applied.

3. Conclusions

3.1. Optimum conditions for the measurement of PPO activity on chlorogenic acid substrate

The formation, at pH 6.2 and 30 °C, of coloured compounds from chlorogenic acid by potato PPO occurs according to zero-order kinetics during the first 5 to 10 min of observation only (Fig. 1). This is attributed partly to enzyme inactivation by covalent coupling with reaction products near the active site (WHITAKER, 1972) and partly to secondary reactions of non-enzymatic character (formation of polymerization products of the oxidized diphenol). It is, therefore, advisable to restrict measurements to the shortest possible period taking OD readings at shorter intervals. It would be equally expedient to reduce the manipulation time of 3 min (para. 2.1) to the minimum. Accuracy and reliability of measurements could, probably, be highly increased by applying an automatic reaction rate analyzer.

Data from the literature report on even shorter linear reaction periods (1 min for apple homogenates at pH 6.6) (MACHEIX, 1970).

The different behaviour of polyphenol oxidase on chlorogenic acid and on the synthetic substrate used in previous work appeared both in developing and in applying the assay method. While with pyrogallol a concentration of 0.25 M was necessary to assure substrate saturation (VÁMOS-VIGYÁZÓ *et al.*, 1973c), chlorogenic acid inhibited the enzyme at concentrations above $3.4 \cdot 10^{-2}$ M (Fig. 2).

MACHEIX (1970) found $1.40 \cdot 10^{-2}$ M to be the lowest concentration of chlorogenic acid ensuring substrate saturation in activity measurements carried out with apples. This concentration is of the same order as the one

found to yield the highest reaction rate in the present study. The author cited above did not observe substrate inhibition up to concentrations of $5 \cdot 10^{-2}$ *M*, whereas the results presented here show a marked decrease in reaction rate at a substrate concentration of $5.1 \cdot 10^{-2}$ *M*.

It must be assumed that the concentration threshold of substrate inhibition depends not only on the substrate, but also on the source of enzyme. Substrate inhibition has been reported (WHITAKER, 1972) for polyphenol oxidase of other products and on other substrates than those dealt with here. The phenomenon was described, among others, for sweet potato PPO with catechol (EIGER & DAWSON, 1949) and for the mushroom enzyme with *p*-cresol as substrate (LONG & ALBEN, 1969).

Enzyme activity of potatoes was found to be approximately proportional to homogenate concentration between the values of 2.0 to 3.2% (w/v) of the latter. The linear section of this plot has been reported to depend on the method of measurement (WHITAKER, 1972): straight lines were obtained with apple PPO by polarographic and spectrophotometric assessment, while chronometric or manometric measurement yielded curves with decreasing slopes at higher enzyme concentrations. Proportionality of activity to homogenate concentration was observed with grapes (ENKELMANN, 1969) only between certain limits of the latter parameter, while the results of MACHEIX (1970) obtained with apples on chlorogenic acid substrate suggest deviation from linearity not only in the high, but in the extremely low range of homogenate concentration, too.

Taking into consideration the *Seluyn*-plots (Fig. 4), together with the relationship between enzyme activity and homogenate concentration (Fig. 3) it seems recommendable to select the value of the latter around 3% (w/v).

3.2. Enzyme concentrations of horticultural products

It is interesting to compare the results presented here with those obtained in this laboratory for the same products with a similar assay method using pyrogallol as substrate (VÁMOS-VIGYÁZÓ *et al.*, 1972, 1973c; VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1973a, 1974).

Comparison is facilitated by the fact that the same arbitrary definitions of unit enzyme activity and of enzyme concentration, resp., were used with both methods.

Enzyme concentrations of potatoes of the variety *Gül baba* (from the years 1971 and 1972) as assessed on pyrogallol were found to vary betwen 0.40 and 0.66 kU g^{-1}. The values obtained on chlorogenic acid were nearly twice as high (Table 3). These findings are in disagreement with those published by SCHWERDTFEGER (1970) according to whom pyrogallol and chlorogenic acid are substrates of similar availability for potato PPO.

Enzyme concentrations of apples obtained on pyrogallol substrate were, in most cases, lower than those presented for the same varieties in Table 3. The values in kU g^{-1} as determined at pH 6.2 ranged from 0.32 to 0.59 for *Jonathan* (1971–1973), from 0.49 to 0.58 for *Golden delicious* (1971–1973), from 0.52 to 0.60 for *Starking* (1971–1973), and from 0.48 to 0.53 for *Staymared* (1972–1973). Enzyme concentrations of *Jonared* (1972) and *Jonadel* (1973) were 0.57 and 0.58, resp. As can be seen, differences between the varieties were less marked on pyrogallol than on chlorogenic acid substrate.

Contrary to the findings presented here, on pyrogallol substrate above varieties had measurable activities at pH 4.0, too, ranging from 0.02 to 0.15 kU g^{-1}.

Pears of the variety *Alexander* were found to have, of all the products investigated, the highest enzyme concentration on chlorogenic acid. In measurements of potential enzymic browning by a spectrophotometric reflectance method (VÁMOS-VIGYÁZÓ *et al.*, 1973b) chlorogenic acid was found to be an excellent substrate for pear PPO: the initial browning rate of pear slices dipped in a $5.6 \cdot 10^{-2}$ *M* chlorogenic acid solution was higher than that observed under similar conditions with a 7.9 M^{-1} pyrogallol solution.

Chlorogenic acid was reported to be a poor substrate for peach PPO (WHITAKER, 1972). This may account for the fact that – apart from one of the lots of *Jonathan* apples – the lowest enzyme concentrations established in this study were those obtained for peaches. On pyrogallol substrate the following values were found for the same varieties: 0.58 and 1.54 kU g^{-1} at pH 4.0 (1973), 0.75–1.00 and 1.12–1.26 kU g^{-1} at pH 6.2 (1972, 1973) for *Ford* and *Elberta*, resp. The great variations in enzyme concentrations as established at pH 6.2 are due to differences in maturity of the lots investigated (VÁMOS-VIGYÁZÓ *et al.*, 1972). It is interesting that on pyrogallol substrate PPO concentration was higher in the variety *Elberta*, while on chlorogenic acid the order was reversed.

For apricots, enzyme concentrations on pyrogallol (1.24–1.65 kU g^{-1}) were found to be considerably lower than on chlorogenic acid substrate.

The examples cited demonstrate the difference in substrate specificity of polyphenol oxidase from various sources.

3.3. Relationship between PPO activity and pH

The pH dependence of PPO was found to be essentially similar in the two peach varieties, but markedly different in potatoes. The pH activity curve as established on pyrogallol substrate for the latter product bears some similarity to that presented in Fig. 5: activity at pH 5.0 was below the limit of detectability on both substrates (VÁMOS-VIGYÁZÓ *et al.*, 1973c, VÁMOS-VIGYÁZÓ & KISS-KUTZ, 1974). This shall be particularly stressed here, because, accord-

ing to several authors (ALBERGHINA, 1964; PATIL & ZUCKER, 1965), the enzyme as purified from potatoes has its activity maximum on chlorogenic acid substrate at pH 5.0. The same pH was found to be the optimum for the chlorogenic acid oxidizing enzyme prepared from egg-plant (SAKAMURA *et al.*, 1966).

Activity maxima were established for PPO from various sources at various pH values between 5 and 8, depending on the substrate (VÁMOS-VIGYÁZÓ & BALÁZS-SPRINCZ, 1970; ASGHAR & SIDDIQUI, 1970). *E.g.* a preparation obtained from cranberries was found to be most active at pH 7.0 and to loose activity abruptly in both the acidic and the alkaline region (CHAN & YANG, 1971).

On the other hand, browning (MACHEIX, 1970) as well as PPO activity of apple homogenates on pyrogallol substrate (VÁMOS-VIGYÁZÓ *et al.*, 1972) yielded curves very similar to those obtained for peaches and potatoes in this study.

Thus, it must be assumed that the effect of pH on activity is different for purified enzyme preparations and for the cell-bound enzyme – a phenomenon that ought to be given more close attention.

Summarizing the results discussed it can be said that the activity assay method lends itself to the determination of PPO concentration in a wide range of horticultural products as well as to the study of some characteristics of the enzyme as present in tissue homogenates of fruits and vegetables.

Literature

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CONTENTS

Index: 26.039

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DETERMINATION, BY REDUCTION, OF THE RED AND TOTAL PIGMENT CONTENT IN PAPRIKA PRODUCTS.

M. BARANYAI and J. SZABOLCS

(Received May 3, 1975)

A spectrophotometric method for determining the red and total pigment content in ground paprika and oleoresins has been developed.

The method is based on the reduction, by $NaBH_4$ of the red polyene ketones in ground red paprika. The reaction was found to be quantitative, causing in the spectrum of the pigment mixture a modification suitable for measuring the optical density (OD) proportional to the red components of the pigment. At the same time the spectrum of the reduced pigment facilitates a more exact determination of OD for the calculation of the total pigment content.

The accuracy of determination of pigment concentration was increased by finding OD factors accounting for the presence of isomers.

When determined by the reduction method, ten native paprika products were found to have a pigment content about 20–25% higher than their *Benedek* number.

The method was checked by analysing model samples of known pigment concentration. The difference in concentration between the measured and the true values did not exceed the error inherent in spectrophotometry.

The various preparations of ground paprika and oleoresins are valuable not only as seasonings, but also for their colouring quality. The commercial value of these products is mainly dependent on their colouring capacity, therefore the determination of their pigment concentration is of great interest. It would be expedient to use standardized grading all over the world, however, the existence of a wide variety of methods presents an obstacle to this.

Earlier the pigment content was determined by subjective colorimetry and photometry (LOWENSTEIN & DUNN, 1910; MOSTER & PRATER, 1952). Today spectrophotometry is used mainly, but the OD figures obtained are still converted by subjective factors into empirical colour values (SHUSTER & LOCKHART, 1954; MOSTER & PRATER, 1957a; 1957b; 1957c; ASTA ANALYTICAL METHODS, 1959; SANCHO & NAVARRO, 1956). The values thus derived are suitable for comparative evaluation, however, they give little information as to the true pigment concentration. It is mainly in Central European countries that optical density values are used for measuring pigment concentration (BENEDEK, 1958; POHLE & GREGORY, 1960; HUNGARIAN STANDARD, 1962). These methods are based on a calibration curve prepared with a selected component of the pigment which is used for determining the total pigment concentration. However, this method only gives approximate results, because the

spectrophotometric characteristics of the components [wave-length of maximum absorption (λ_{max}) and maximum molar absorptivity (ε_{max}, molar extinction coefficient)] differ.

The differences are caused mainly by the circumstance that, apart from the red components, the pigment contains yellow ones as well. Recent investigations have shown that in pigments isolated from various paprika samples the proportion of red and yellow components varies (VINKLER & KISZEL-RICHTER, 1972; HARKAY-VINKLER, 1974). Since in the colouring of foods red pigments are of decisive significance it is desirable that besides the total pigment content, the quantity of red components should also be determined.

Our investigations yielded a simple and rapid spectrophotometric method for the determination of the red and total pigment content of paprika and oleoresin products.

1. Materials and methods

1.1. Materials

All chemicals were of analytical grade. Capsanthin dipalmitate was prepared from capsanthin (ex. *Capsicum annuum* var. *lycopersiciforme rubrum*) according to ZECHMEISTER and CHOLNOKY (1940). Melting point: 96°C; λ_{max} (ε_{max}) in benzene: 514 nm (93 000), 488 nm (113 000) and 460 nm (78 000).

Capsorubin dipalmitate was gained from capsorubin (ex. *Capsicum annuum* var. *lycopersiciforme rubrum*; ZECHMEISTER & CHOLNOKY, 1940). Melting point: 97°C; λ_{max} (ε_{max}) in benzene: 524 nm (118 000), 489 nm (130 000), and 455 nm (90 000).

Zeaxanthin diacetate was prepared by acetylation of zeaxanthin (ex. *Physalis alkekengi;* SZABOLCS & TIMÁR, 1973). Melting point: 140°C; λ_{max} (ε_{max}) in benzene: 495 nm (108 000), 464 nm (124 000) and 439 nm (88 000).

Lutein diacetate was obtained by the acetylation of lutein (ex. *Ranunculus acer*; SZABOLCS & TIMÁR, 1973). Melting point: 168°C; λ_{max} (ε_{max}) in benzene: 487 nm (113 000), 457 nm (126 000) and 432 nm (85 000).

β-carotene (FLUKA Co.). Melting point: 182°C; λ_{max} (ε_{max}) in benzene: 495 nm (110 000), 466 nm (125 000) and 438 nm (87 000).

The ground paprika samples and oleoresins used in the experiments were commercial products of the KALOCSA FACTORY OF THE PAPRIKA AND CANNING INDUSTRIES, made available by the RESEARCH INSTITUTE OF THE CANNING AND PAPRIKA INDUSTRIES, Budapest.

1.2. Methods

To extract the pigment content of ground paprika, a method as described by BENEDEK (1958) was applied. For spectrophotometry the extracts were diluted 1 : 9 or 1 : 4.

Of the oleoresins 0.5% solutions were prepared in benzene. Depending on their pigment content these solutions were then diluted 1 : 7 or 1 : 80.

The spectra were run in a *Perkin-Elmer* 402 spectrophotometer in the 270–590 nm range.

To obtain reduction, approximately 10^{-5} *M* benzene solutions of the model compounds and of the pigments were used. Five ml of these solutions were mixed with 5 ml 96% ethyl alcohol and then saturated with finely powdered $NaBH_4$ (CARLO ERBA or REANAL). The substrate was then kept at room temperature in a dark place for 40 min. Subsequently a NaOH tablet was added. Prior to spectrophotometry it was filtered through a fluted analytical grade filter paper. The first 3 ml were discarded and the required amount was filtered directly into the cuvette.

Iodine isomerization was carried out according to ZECHMEISTER (1962).

2. Results

2.1. Analysis of the pigment spectrum

Generally, spectrophotometry is used to determine the pigment concentration in ground paprika, because the pigment is a mixture of carotenoids having a characteristic visible spectrum (Fig. 1).

The ε_{max} values belonging to the carotenoid pigments are in the order of magnitude of 100 000. Thus in the spectrophotometry of paprika, pigment solutions of 10^{-5} *M* may be used, for which the *Bouguer–Lambert–Beer* law is valid.

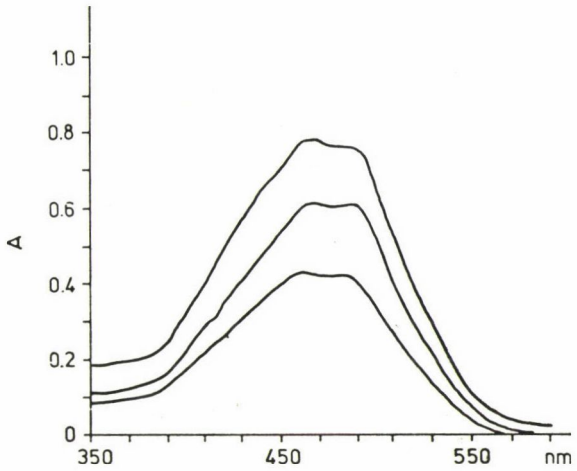

Fig. 1. Spectra in benzene of the pigments, extracted according to BENEDEK (1958), of three commercially available ground paprika samples

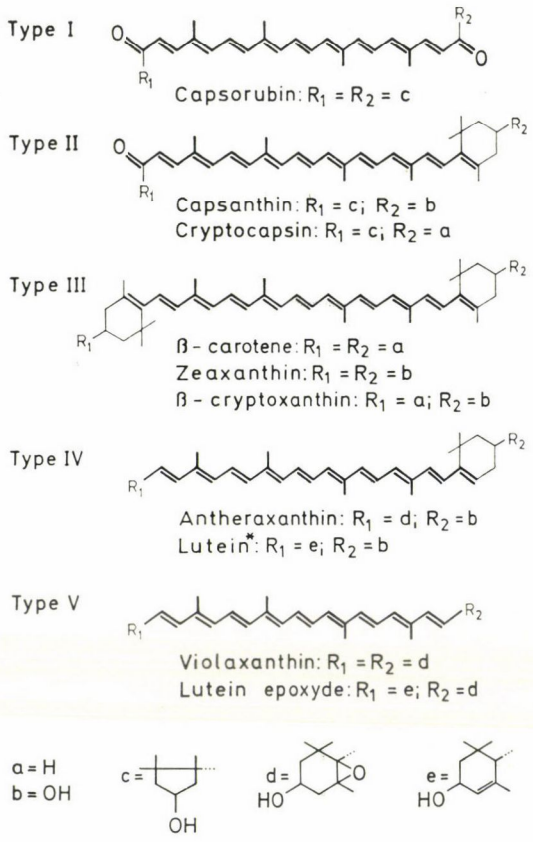

Fig. 2. Types of chromophores of the pigment components

According to a study of CHOLNOKY and co-workers (1955) the paprika pigment consists of about 20 different carotenoids, including the isomers. However, from the point of view of spectrophotometry these may be divided into 5 types of chromophores (Fig. 2).

Chromophores I and II represent the red colouring substance consisting of three components and their isomers. Seven yellow components belong to chromophore types III, IV and V. The chromophores of aurochrome, mutatochrome and mutatoxanthin differ from the above-enumerated types. However, the total amount of these three components is below 2% according to CHOLNOKY and co-workers (1955). Paprika pigment mainly contains chromophores of two types: capsanthin, belonging to type II and β-carotene, β-cryptoxanthin and zeaxanthin, belonging to type III. One spectrum of each of the chromophore types listed above is shown in Fig. 3.

* In accordance with the literature it was found in this study that the pigment contained some lutein (VINKLER & KISZEL-RICHTER, 1972; HARKAY-VINKLER, 1974)

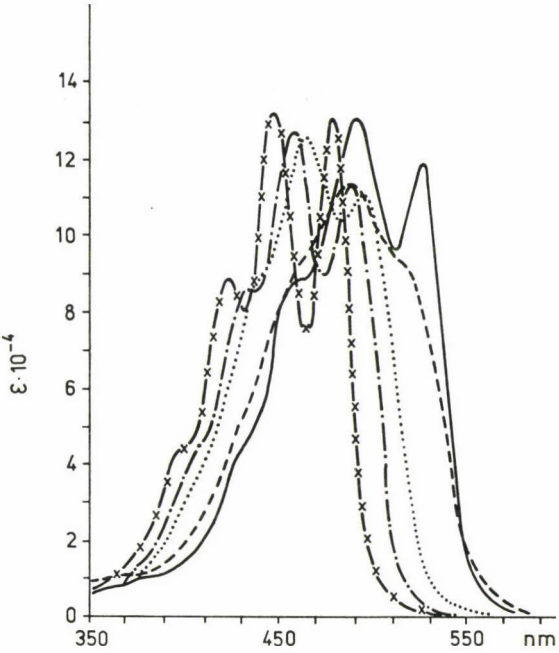

Fig. 3. Molecular absorption spectra in benzene of the pigment components with various types of chromophores. ——— Type I: capsorubin (λ_{max}: 524, 489, 455); — — — Type II: capsanthin (λ_{max}: 514, 488, 460); ····· Type III: β-carotene (λ_{max}: 495, 466, 438); — · — · — Type IV: antheraxanthin (λ_{max}: 489, 458, 434); — × — × — Type V: violaxanthin (λ_{max}: 483, 453, 424)

As seen from the λ_{max} values in Fig. 3, the red components absorb light at longer wave-lengths, while the yellow components in a shorter range. (As may be seen in the spectra of the yellow components, with the decrease of double bonds in the chromophores the absorption maxima appear at shorter wave-lengths.) However, the differences between the absorption maxima are slight, therefore the spectra greatly overlap. Thus it is only above 530 nm that the spectrum of the pigment contains OD values characteristic of the red components.

Below 530 nm all OD values are characteristic of the total pigment because at every wave-length they are composed of the optical density values of the red and yellow components. In Fig. 4 the development of the spectrum of red paprika from its components is illustrated. From the five main pigment components stock benzene solutions of $0.6 \cdot 10^{-5}$ M concentration were prepared. The models were obtained by mixing aliquot volumes of these solutions.

Wave-length 496 nm, as indicated by an arrow in Fig. 4, was selected by BENEDEK (1958) to yield the OD value proportional to the total pigment content. The OD value thus obtained is used to calculate the concentration with the help of the optical density factor for capsanthin while, as seen in the

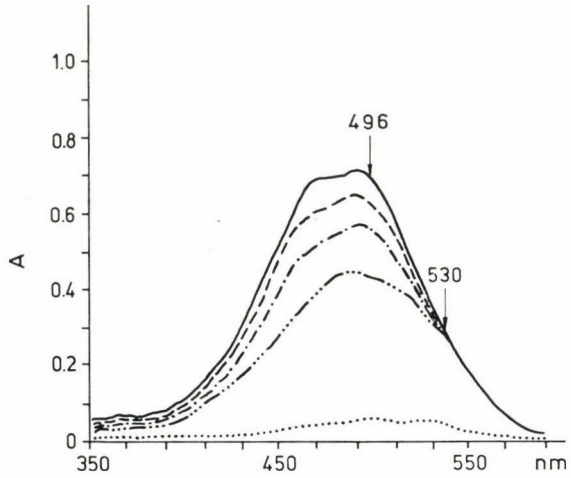

Fig. 4. Development of the spectrum of the red paprika model from its components. ····· A+benzene (1 : 9); — · · · — A+B+benzene (1 : 5 : 4); — · — · — A+B+C+benzene (1 : 5 : 2 : 2); — — — A+B+C+D+benzene (1 : 5 : 2 : 1 : 1); ——— A+B+C+D+E (1 : 5 : 2 : 1 : 1) (A = capsorubin stock solution; B = capsanthin stock solution; C = β-carotene stock solution; D = zeaxanthin stock solution; E = lutein stock solution)

figure, at this wave-length the yellow components show a substantial absorption, too. At 496 nm, however, the molar absorptivity for the yellow components is lower than that for capsanthin (Fig. 3). Thus it follows that the pigment content, determined according to *Benedek*'s method, is lower than the true value. Practically the same applies to other methods of pigment determination.

When comparing the spectra of the carotenoids of red paprika of various types it may be concluded that the absorbance values directly read are not suitable for calculating either the red or the total pigment content.

2.2. Reduction by sodium borohydride using model solutions

Comparison of the chromophores of the paprika carotenoids in Fig. 2 shows that the red chromophores (I and II), in contrast to the yellow ones (III, IV and V), contain conjugated oxo-groups. Thus, in spite of the same number of double bonds, due to the bathochromic shift caused by conjugated oxo-groups, the red components are absorbing in a longer wave-length range (Fig. 3).

Consequently, if an oxo-group selective reducing agent is added to the pigment solution the number of conjugated double bonds of the red components is reduced and a decrease of OD characteristic of the red components will be observed in the pigment spectrum at a longer wave-length.

On the basis of Fig. 2 it may be concluded that the selective and quantitative reduction of type I and type II red components yields an equal amount of yellow components containing type IV and type V chromophores. Thus the OD characteristic of the total pigment content may be determined from the spectrum of a spectroscopically more homogeneous mixture.

A capsanthin dipalmitate model was used to develop this simple method. suitable for routine measurements, capsanthin being the chief oxo-carotenoid in paprika pigment. A 1 : 1 mixture of 96% ethyl alcohol and benzene, saturated with $NaBH_4$, was found to give a satisfactory reaction velocity at room temperature. When reacting with $NaBH_4$, capsanthin dipalmitate (VI) is converted into capsanthol dipalmitate (VII) (Fig. 5) and the red solution turns yellow.

The reduction may be followed by spectrophotometry. The spectra of the original substance and of the reduced product are shown in Fig. 6.

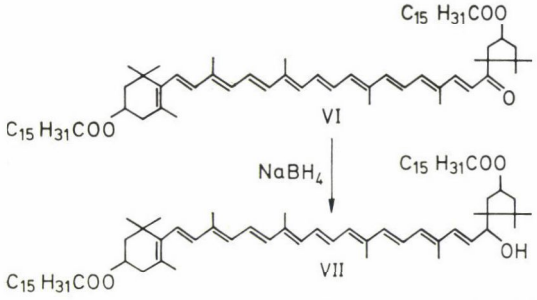

Fig. 5. Reduction of capsanthin dipalmitate (VI) to capsanthol dipalmitate (VII) by $NaBH_4$

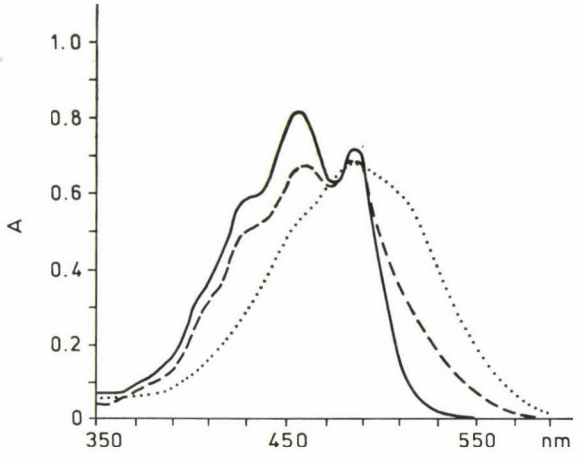

Fig. 6. Spectroscopic follow-up of the reduction by $NaBH_4$ of capsanthin dipalmitate in 96% ethyl alcohol–benzene mixture (1 : 1). ····· Capsanthin dipalmitate (0 min); — — — mixture of capsanthin dipalmitate and capsanthol dipalmitate (in the 20th min); ——— capsanthol dipalmitate (in the 40th min)

As a result of the reduction of the oxo-group the chromophore became shorter and the spectrum of the product moved to a shorter wave-length range. As shown by the spectrum at the 20th min of the reduction, capsanthin dipalmitate is still present in the solution. The spectrum taken at the 40th min is identical with the spectrum of the authentic capsanthol dipalmitate (Fig. 14c), which proves that the reduction is completed. The reaction time of 40 min is valid for solutions of a pigment concentration of 10^{-5} M and saturated relative to $NaBH_4$, at room temperature.

Selectivity and completeness of the reduction were checked experimentally. Three capsanthin dipalmitate solutions of different concentrations were reduced and their concentrations determined by spectrophotometry. Molar concentrations of the original samples and those of the reduced products corresponded in all three samples; the slight differences did not exceed the error inherent in spectrophotometry (Table 1).

Selectivity and completeness of the reduction of the other red component of the pigment, capsorubin dipalmitate (VIII) containing the type II chromophore, were similar. On reduction with $NaBH_4$ this component was converted into capsorubol dipalmitate (IX).

The spectra (Fig. 8) of the original substance and the reduction product show a difference of 50 nm in their absorption maxima, proving thereby the reduction of both oxo-groups.

A further proof of selectivity and completeness is that, in spite of the presence of $NaBH_4$ in excess, the spectrum of capsorubol dipalmitate did not change during the 24 hours following the completion of the reaction.

Table 1

Checking of the quantitative reduction of capsanthin dipalmitate solutions of known concentration by spectrophotometric measurement of the concentration in the reduced solutions

Samples	Weight of capsanthin dipalmitate, mg	Concentration of capsanthin dipalmitate solutions		OD of capsanthol dipalmitate solutions at 452 nm (*a*)	Concentration of capsanthol dipalmitate solutions	
		mg l^{-1}	M (M_W = 1 060)		mg l^{-1}	M (M_W = 1 062) (*b*)
1.	0.752	7.52	$7.09 \cdot 10^{-6}$	0·93 0.92	7.59	$7.15 \cdot 10^{-6}$
2.	0.810	4.05	$3.82 \cdot 10^{-6}$	0.52 0.52	4.08	$3.84 \cdot 10^{-6}$
3.	1.098	5.49	$5.18 \cdot 10^{-6}$	0.67 0.68	5.47	$5.15 \cdot 10^{-6}$

(*a*) Two parallel reductions of capsanthin dipalmitate were carried out
(*b*) Molecular absorption at 452 mm of capsanthol dipalmitate in the 1 : 1 mixture of 96% ethanol and benzene

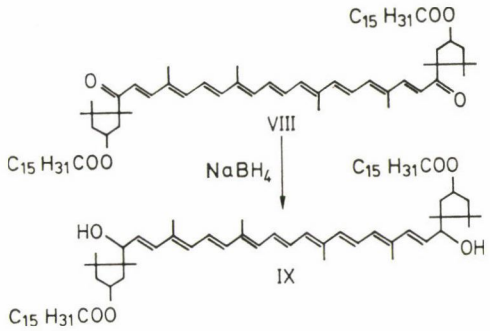

Fig. 7. Reduction of capsorubin dipalmitate (VIII) to capsorubol dipalmitate (IX) in response to $NaBH_4$

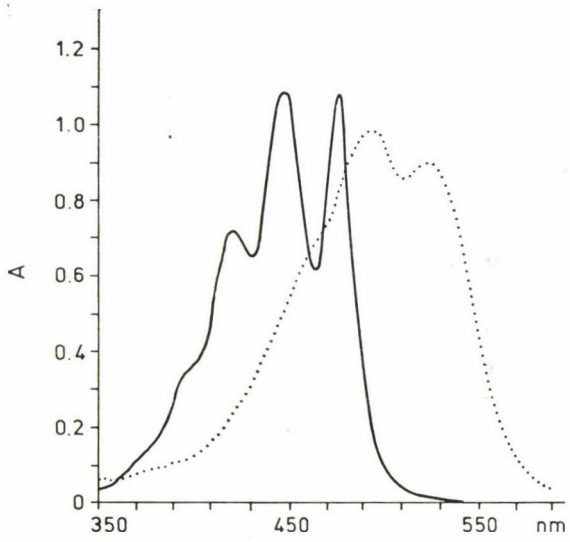

Fig. 8. Spectra of capsorubin dipalmitate (·····) and capsorubol dipalmitate (———) in 96% ethyl alcohol–benzene mixture (1 : 1)

2.3. Reduction of the paprika pigment with $NaBH_4$

After the model experiments, the paprika pigment was exposed to reduction. From commercially available ground paprika the pigment was extracted according to BENEDEK (1958). (0.500 g ground paprika was extracted with 100 ml benzene in a laboratory shaker for 30 min. After 5 min of sedimentation an aliquot of 10 ml was removed.) The concentration suitable for spectrophotometry was adjusted by dilution, and reduction was carried out with $NaBH_4$ as described for the model experiments, in the 1 : 1 mixture of 96% ethyl alcohol and benzene. As in the model experiments the difference between the spectra of the native pigment and the reduced pigment (Fig. 9) was characteristic.

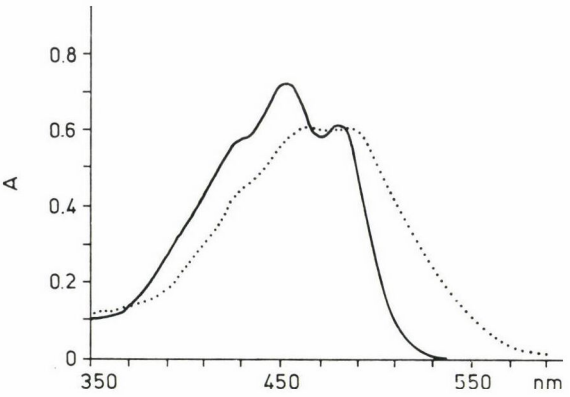

Fig. 9. Spectra of the ground paprika pigment in the native (·····) and in the reduced state (———) in 96% ethyl alcohol–benzene mixture (1 : 1)

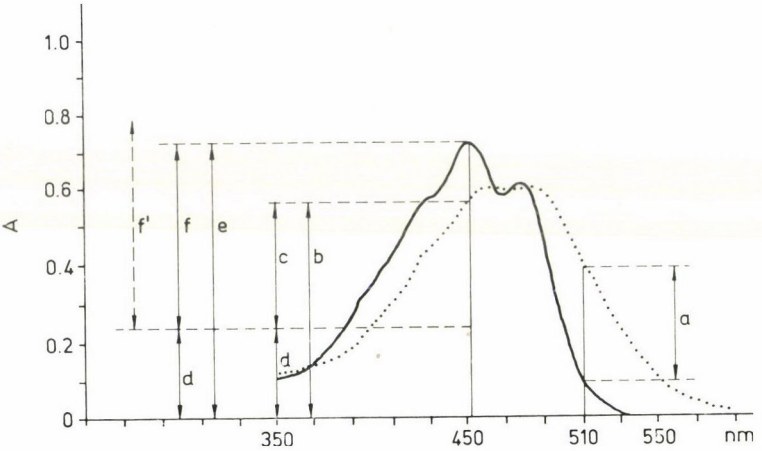

Fig. 10. Spectroscopic checking of the quantitative reduction of paprika pigment in 96% ethyl alcohol–benzene (1 : 1). ····· Spectrum of the native pigment; ——— spectrum of the reduced pigment. a. OD at 510 nm proportional to the quantity of the red component; b. OD at 455 nm of the native pigment, composed of c and d (b = c+d); c. OD of the red components in the native pigment, which in the knowledge of "a", can be calculated from the molar absorption curve at 455 nm; d. OD of the yellow components in the native pigment at 455 nm (d=b−c); e. OD of the reduced pigment at 455 nm, composed of d and f (e = d+f); f. OD of the yellow components formed upon the reduction of the red components (f = e−d); f'. OD of the yellow components formed theoretically upon the reduction of the red components showing absorption "a", calculated using the molar absorption factor of the all-*trans* models

However, during evaluation of the spectra by means of chromophores it appeared that the presence of isomers had not been taken into account. When the red components (a) and the yellow components formed upon reduction (f) were measured at the wave-lengths indicated in Fig. 10, their amounts — unlike in the model experiments — were not identical, but consistently lower than expected theoretically (f') (Fig. 10).

Since this error was not observed with the paprika models investigated (there was no difference between the theoretical and the measured quantities) the composition of the pigment was analysed again and it was found that, in contrast to the models, a substantial amount of *cis*-carotenoids was present. These compounds contain the same number of double bonds as their all-*trans*-isomer, but one or more double bonds in the conjugated chain in their chromophore is in *cis* position. In their molar absorption curve, this causes a difference dependent on the position of the *cis* double bond. As an example, β-carotene (X), containing type III chromophore, and its central *cis*-isomer (XI) should stand here (Fig. 11).

Their molar OD curve is illustrated in Fig. 12.

The reduction of the molar OD value of the *cis*-isomers and the shift in λ_{max} may cause a substantial error in the determination of concentration. Isomerization experiments have shown, however, how the *cis*-isomers present in the pigment may be accounted for in quantitative evaluation. It is known

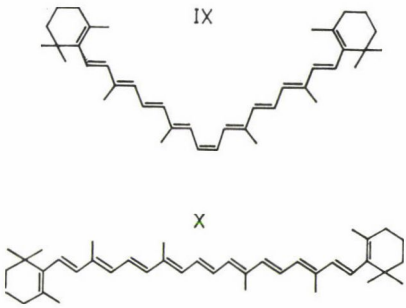

Fig. 11. β-carotene (X) and its central *cis*-isomer (XI)

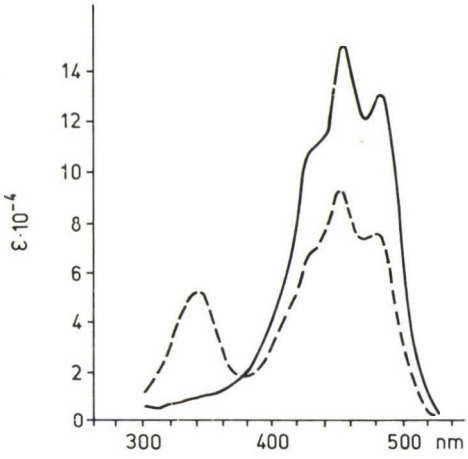

Fig. 12. Molar absorption curves in benzene of all-*trans*-β-carotene (———) and 15-*cis*-β-carotene (— — —) (JAFFE & ORCHIN, 1962)

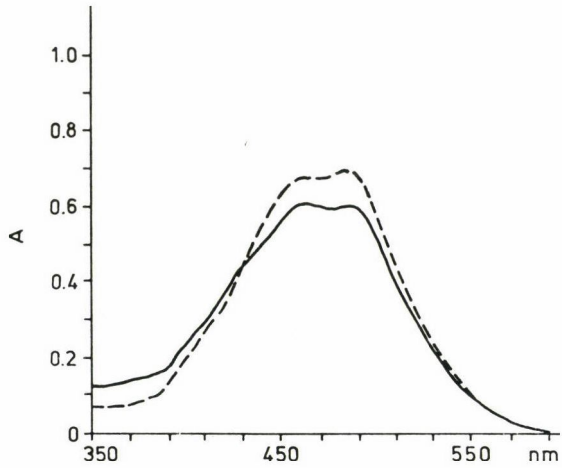

Fig. 13. Spectrum in benzene of the all-*trans* pigment model (— — —) and its iodine catalyzed equilibrium mixture (———)

that in the benzene solution of carotenoids, under constant conditions, an isomeric equilibrium mixture is formed by iodine-catalysis. This contains a predetermined proportion of the all-*trans* compound and its *cis*-isomers (ZECHMEISTER, 1962). The all-*trans* compound, shown in Fig. 4, was used in an iodine isomerization experiment and the result, according to expectations, showed the absorption values of the isomeric equilibrium mixture to be lower than that of the all-*trans* mixture (Fig. 13).

In contrast, when the native pigment containing *cis*-isomers as well was iodine isomerized, its spectrum did not show any change. No change could be observed in the spectra of the reduced native and isomerized pigments either. The same result was obtained upon isomerization of the 10 pigment mixtures listed in Table 3.

Thus it may be concluded that the isomeric mixtures of native pigments and the isomeric equilibrium mixtures are of almost the same composition, or the slight differences in composition between them do not cause variations in their spectra. Therefore the presence of isomers can be taken into account in determining the concentration by calculating the OD factors from the molar equilibrium absorbance curves of the iodine-isomerized equilibrium mixture of model compounds and not from the all-*trans* compounds.

2.4. Determination of the red components

After comparison of the spectra of the iodine-catalyzed equilibrium mixture of capsanthin and that of capsorubin dipalmitate (Fig. 14a) with the spectra of the native and the reduced pigment (Fig. 14b) wave length 510 nm was

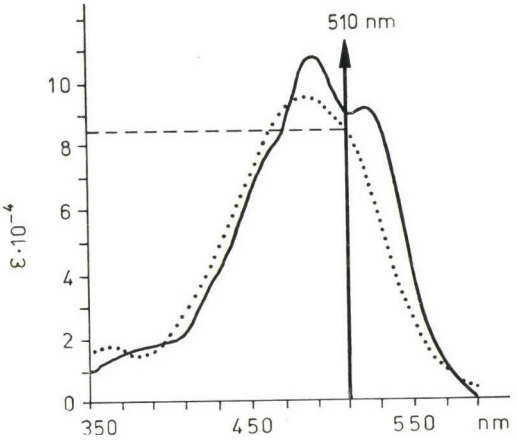

Fig. 14a. Molar absorption curve of the iodine-catalyzed equilibrium mixture of capsanthin dipalmitate (·····) and capsorubin dipalmitate (———) in 96% ethyl alcohol and benzene (1 : 1)

selected for measurement of the OD proportional to the quantity of the red component.

This is one of the maxima (shoulder) in the spectrum of capsanthin dipalmitate, however, it is even more important that the molar absorptivities of capsanthin and capsorubin dipalmitate are nearly identical at 510 nm. Thus for the molar absorptivities of the red components at 510 nm the value 85 000 pertinent to capsanthin may be used. The slight deviation of the spectrum of capsorubin dipalmitate may be neglected, since this compound forms only about 5–10% of the pigment.

It is seen in Fig. 14b that the difference in absorbance between the native

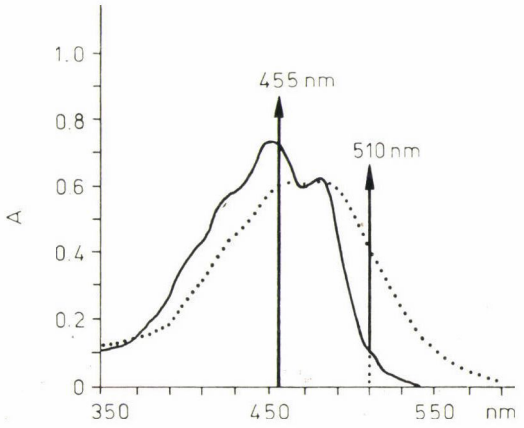

Fig. 14b. Spectrum in 96% ethyl alcohol–benzene (1 : 1) of the native (·····) and the reduced pigment (———)

and reduced pigments is characteristic of the red components at 510 nm. This is demonstrated by the difference spectrum in Fig. 15.

Differential measurement is advantageous, because the selected value at 510 nm is at the maximum of the difference spectrum and the OD value thus measured differs only slightly from the OD truly proportional to the red components, and the difference may be eliminated by the application of a correction factor (Fig. 16).

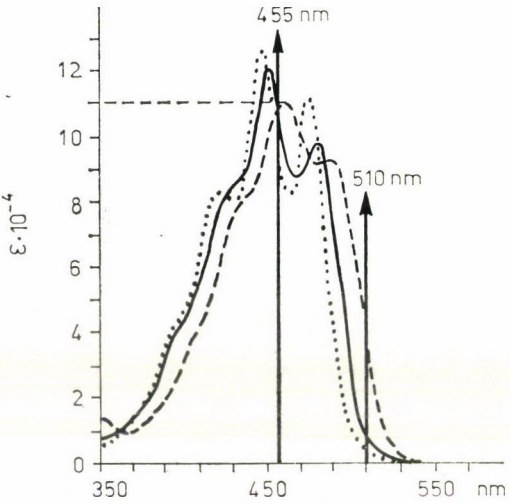

Fig. 14c. Molar absorption curve in 96% ethyl alcohol–benzene (1 : 1) of the iodine catalyzed equilibrium mixture of β-carotene (— — —), capsanthol dipalmitate (———) and capsorubol dipalmitate (·····)

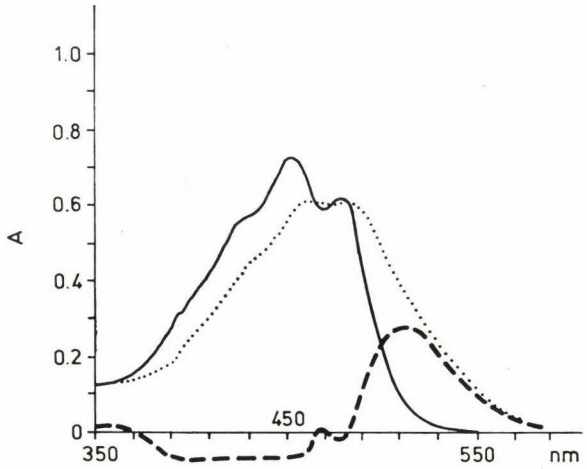

Fig. 15. Difference spectrum (— — —) in 96% ethyl alcohol–benzene (1 : 1) of the native pigment (·····) related to the reduced pigment (———)

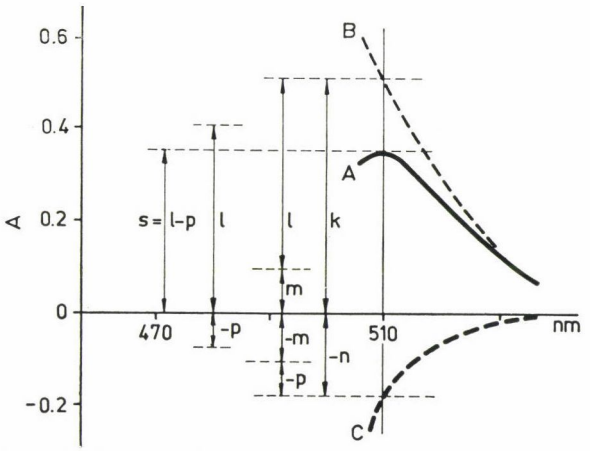

Fig. 16. Analysis of the difference value as measured at 510 nm. A = difference spectrum between 500 nm and 550 nm; B = spectrum of the native pigment between 500 nm and 550 nm; C = spectrum of the reduced pigment between 500 nm and 550 nm in the case of differential measurement; k = OD of the native pigment at 510 nm, consisting of two components: k = l+m; l = OD of the red components of the native pigment at 510 nm; m = OD of the yellow components in the native pigment at 510 nm; −n = OD of the reduced pigment at 510 nm, in the case of differential measurement, consisting of two components: −n = (−m) + (−p); −p = OD of the yellow components formed upon reduction of the red components at 510 nm, in the case of differential measurement; s = OD as measured on the difference spectrum, being less than the true OD of the red pigment: s = l−p

Figure 16 shows that, essentially, the measurable difference (s) is decreased by only that portion of the absorption at 510 nm of the reduced pigment (in the case of differential measurement, −n) which is caused by the yellow components formed during the reduction of the red pigment (−p). The OD proportional to the yellow components originally present in the pigment (m) becomes compensated for during differential measurement (−m+m = 0). It follows from the molar OD curves of the red components and their reduced products (Figs. 14a and 14c) that differential measurement at 510 nm gives about 93 % of the true OD and thus the value of the correction factor is 100/93 = = 1.07.

In calculating the concentration in mg l^{-1} (ppm), as used in practice, the average molecular weight of the red components was used. Taking into account an average composition of 10 % capsorubin (M_W = 600) and 90 % capsanthin (M_W = 584) the molecular weight was 586. Using the 1 : 1 mixture of 96 % ethyl alcohol and benzene, the concentration of the red components is calculated by the following equation:

$$c(\text{mg } l^{-1}) = \frac{A_{510} \cdot 1.07 \cdot 586 \cdot 1\,000}{85\,000}$$

where

A_{510} = OD of the native pigment, in a cuvette of 1 cm, relative to the reduced pigment;

1.07 = correction factor;
586 = average molecular weight of the red components;
85 000 = molar absorptivity of the red components at 510 nm.

2.5. Determination of the total pigment content

The reduction of native paprika pigment facilitated not only the determination of the red components, but also a more exact measurement of the total pigment content. It has been mentioned before that the reduction gave a more homogeneous spectroscopic mixture composed of yellow components containing type III, IV and V chromophores (Fig. 2). The molar OD curves of the iodine-catalyzed equilibrium mixtures of β-carotene, capsanthol dipalmitate and capsorubol dipalmitate, representing the above three types of chromophores, contain iso-absorption points at 455 nm. The spectrum of the reduced pigment has a maximum at 455 nm (Fig. 14b), therefore the wave-length of 455 nm was chosen for the measurement of the OD of the total pigment content. At this wave-length the average molar absorptivity is 110 000 (Fig. 14c). The average molecular weight of the total pigment was calculated on the basis of the average composition: 50% capsanthin ($M_W = 584$), 10% capsorubin ($M_W = 600$), 10% β-carotene ($M_W = 536$), 10% zeaxanthin ($M_W = 568$), 10% lutein ($M_W = 568$) and 10 % β-cryptoxanthin ($M_W = 552$) and was found to be 575.

The equation used in calculating the total pigment concentration in the (1 : 1) mixture of 96% ethyl alcohol and benzene is as follows:

$$c(\mathrm{mgl^{-1}}) = \frac{A_{455} \cdot 575 \cdot 1\,000}{110\,000}$$

where

A_{455} = OD of the reduced pigment at 455 nm;
575 = average molecular weight of the total pigment;
110 000 = molar OD of the total pigment at 455 nm.

2.6. Practical application of the method based on reduction

The method was checked by the measurement of the pigment concentration in paprika models. Of the main components $0.8 \cdot 10^{-5}$ M solutions were prepared. 400 ml capsanthin dipalmitate and 100 ml capsorubin dipalmitate solution were used to prepare 500 ml red stock solution, and 200 ml β-carotene, 100 ml zeaxanthin diacetate, 100 ml lutein diacetate and 100 ml β-cryptoxanthin acetate to prepare 500 ml yellow stock solution. In the eight different models mixed from the two stock solutions the amounts of red and yellow components were varied, while the total pigment concentration was kept at $0.8 \cdot 10^{-5}$ M. The samples were then isomerized with I_2, reduced and used for measurement by spectrophotometry. As seen in Table 2, the concentrations,

Table 2

Determination of the red and total pigment content in paprika models of known concentration by the reduction method

Serial number of model	Concentration in all-*trans* model			Red pigments upon isomerization		Total pigment content upon isomerization		Difference between true and measured concentrations (Δc) $10^{-5}M$	
	Total pigment	Red components		A_{510}	c 10^{-5} *M*	A_{455}	c 10^{-5} *M*		
	10^{-5} *M*	10^{-5} *M*	mole %					red	total
1.	0.80	0.80	100	0.63	0.79	0.90	0.82	−0.01	+0.02
2.	0.80	0.64	80	0.52	0.66	0.88	0.80	+0.02	Ø
3.	0.80	0.56	70	0.44	0.55	0.88	0.80	−0.01	Ø
4.	0.80	0.48	60	0.39	0.49	0.88	0.80	+0.01	Ø
5.	0.80	0.40	50	0.32	0.40	0.89	0.81	Ø	+0.01
6.	0.80	0.32	40	0.25	0.32	0.90	0.82	Ø	+0.02
7.	0.80	0.16	20	0.12	0.15	0.89	0.81	−0.01	+0.01
8.	0.80	—	—	—	—	0.88	0.80	—	Ø

Table 3

Determination of the red and total pigment contents in three commercial ground paprika samples (PP) and seven oleoresins (OR) by Benedek's method and by the reduction method

Sample	*Benedek* number (*a*) g kg^{-1}	Colour index (*a*)	*Benedek* number (*b*) g kg^{-1}	Results of the reduction method		Percentage of red components %
				Total pigment (*b*) g kg^{-1}	Red components (*b*) g kg^{-1}	
PP 1	3.05	—	3.04	3.86	2.40	62.2
PP 2	4.07	—	4.10	5.08	3.28	64.6
PP 3	special	—	6.66	8.32	4.80	57.7
OR 1	—	8 000	3.40	4.08	3.00	73.2
OR 2	—	25 000	14.50	17.40	12.00	69.0
OR 3	—	30 000	17.20	21.20	13.20	62.3
OR 4	—	40 000	24.10	30.00	18.00	60.0
OR 5	—	60 000	36.00	42.80	26.80	62.6
OR 6	—	80 000	54.60	67.00	48.00	71.8
OR 7	—	100 000	68.40	86.00	52.50	61.1

(*a*) Results of quality control in the factory
(*b*) Results of laboratory determinations

as established after reduction, correspond practically with the true concentrations, the difference not exceeding the error of spectrophotometry.

In the last series of experiments the total pigment content and the red components were determined by the reduction method in three different paprika samples and seven oleoresin samples. For comparison, the total pigment content was determined also by *Benedek*'s method. The results are presented in Table 3. It can be seen that the results obtained by the reduction method are 20–25% higher than those obtained by *Benedek*'s method. The difference is due to the fact that in the latter method the OD values obtained at 496 nm are considered as capsanthin, while the yellow components and the reduction in OD as caused by the presence of isomers are disregarded.

3. Conclusions

A simple method was developed for the determination of the total pigment content in ground paprika and oleoresins, based on spectrophotometry and suitable for routine tests.

The method is carried out as follows:

1. Weigh 0.500 g ground paprika on an analytical balance.

2. Transfer it quantitatively to a stoppered, preferably dark bottle, previously filled with N_2 and add 100 ml of benzene.

3. Extract the pigment content of the ground paprika by shaking the bottle in a laboratory shaker for 30 min and allow it to settle for 5 min.

4. Transfer 10 ml of the clear solution to a volumetric flask and make it up to 50 ml with benzene. (If the pigment content is low, dilute it to 25 ml.) Mix 10 ml of the diluted benzene solution with 10 ml of 96% ethyl alcohol, shake and divide into two test tubes provided with ground stoppers, each tube containing about 10 ml.

5. Saturate the contents of one of the test tubes with previously powdered $NaBH_4$. Add sufficient reagent to obtain a little undissolved $NaBH_4$ at the bottom.

6. Allow the two solutions to stand for at least 40 min, shaking them from time to time.

7. After 40 min add 1 tablet ($\sim$0.2 g) of NaOH to the solution turned yellow, filter immediately through a fluted analytical grade filter paper into a 1 cm cuvette discarding the first 3 ml. Measure the OD of the solution at 455 nm (A).

8. Pour the native pigment solution into another cuvette and measure its OD relative to that of the reduced solution at 510 nm (B).

9. Use the following equations to calculate the red and total pigment contents (D = dilution):

$$\text{Red pigment in the paprika (mg g}^{-1}) = \frac{B \cdot 1.07 \cdot 586 \cdot 10^3 \cdot D \cdot 2}{85\,000 \cdot 10} \simeq 1.5 \cdot B \cdot D$$

$$\text{Total pigment in the paprika (mg g}^{-1}) = \frac{A \cdot 575 \cdot 10^3 \cdot D \cdot 2}{110\,000 \cdot 10} \simeq A \cdot D$$

In the case of oleoresins the test solution is prepared by diluting 0.500 g oleoresin directly. The procedure is otherwise the same as given in paras. 5–9.

Thanks are due to Miss É. NYERS for technical assistance and to the RESEARCH INSTITUTE OF THE CANNING AND PAPRIKA INDUSTRIES, Budapest, Hungary, for providing the paprika and oleoresin samples.

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THE EFFECT OF PIMARICIN ON THE MICROBIAL FLORA OF COTTAGE CHEESE IN PLASTIC PACKAGING

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The change in the viable cell count and pimaricin activity in cream cottage cheese, prepared at the HUNGARIAN INSTITUTE FOR DAIRYING with brine containing *Delvocide*, the aqueous suspension of 5% pimaricin and 0.6% NaCl, packaged in plastic containers and stored at 10–14°C, was investigated. Comparative studies were made as to the pimaricin sensitivity and lactose degrading capacity of yeast strains isolated from spoiled cream cottage cheese.

The difference in bacterial count between samples of 25 or 250 μg ml^{-1} initial pimaricin concentration in the brine and the control samples was insignificant and changed only slightly during the test period. The viable yeast count in the brine containing 250 μg ml^{-1} pimaricin was lower by 1.5 orders of magnitude already at the beginning of the storage period. During storage the yeast count in the control samples gradually increased, while in the samples containing pimaricin a strong fungistatic, or fungicidal effect manifested itself.

During the approximately 6 weeks long storage period pimaricin penetrated the cottage cheese only to the depth of 1 cm. On storage the activity of the antibiotic decreased and the relative decrease in activity was more extensive in samples with lower initial concentration (25 μg ml^{-1}) than with samples of higher initial concentration (250 μg ml^{-1}).

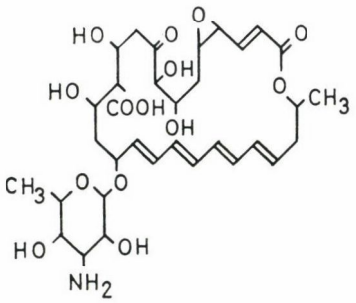
The yeast strains isolated from cream cottage cheese did not show lactose degrading capacity. From the practical point of view the difference in pimaricin sensitivity was insignificant. Measured by the agar-diffusion technique, the minimum inhibitory concentration was of the order of 5–10 μg ml^{-1} pimaricin.

Cottage cheese packed with brine in plastic containers frequently becomes puffed and undergoes spoilage. This is due to the proliferation and gas formation of yeasts inevitably contaminating cottage cheese during processing.

Since the preservatives permitted in Hungary were ineffective or, when administered in an efficient dose, substantially reduced the sensory quality of the product, as proven in experiments carried out at the HUNGARIAN INSTITUTE FOR DAIRYING, Mosonmagyaróvár (PULAY & SZABÓ, 1970), it seemed expedient to investigate the applicability of the strong antifungal antibiotic pimaricin (TRESNER, 1956; KLIS *et al.*, 1959).

Pimaricin is produced by *Streptomyces natalensis* (STRUYK *et al.*, 1958) and it is of amphoteric character, creamy white colour, odourless and practically tasteless. According to its chemical structure it belongs to the group of polyene macrolide antibiotics, shown to have in its molecule a macrocyclic lactone group containing a conjugated tetraene carbon chain (PATRICK *et al.*, 1958 a, b;

BERRY, 1963; OROSHNIK & MEBANE, 1963). The lactone ring is linked to micosamine by an ether bond (CEDER *et al.*, 1963):



The first review of the literature on the utilization of pimaricin in the food industries was given by CLARK and co-workers (1964) and AYRES and co-workers (1964). To our knowledge, WHITEHILL (1956) was the first to use pimaricin to increase the keeping quality of cream cottage cheese. He succeeded in nearly doubling the keeping quality at refrigerator temperature by adding pimaricin to the salt used in processing at a concentration of 10–20 $\mu g\ g^{-1}$. Similarly satisfactory were the results of SHAHANI and co-workers (1959) and NILSON and co-workers (1960) who added pimaricin to the washing water used in cottage cheese manufacture.

MOL (1959) described the good results he obtained by dipping hard cheese in a pimaricin solution of 100 $\mu g\ ml^{-1}$. Very successful were the experiments of HOOGERHEIDE (1964) who coated hard cheese with polyvinyl acetate containing 500–1 000 $\mu g\ ml^{-1}$ of pimaricin. MOL (1966) carried out similar experiments and found that pimaricin penetrated the cheese only to a depth of 0.5–1.0 mm and caused no change in its taste.

LINDGREN and LODIN (1969) found that the application of a dipping solution containing 5–10 mg ml^{-1} pimaricin inhibited mould growth in cheese slices packed in plastic film. They observed also that pimaricin, in contrast to sorbic acid, did not affect the taste of the cheese.

According to the report of a WHO Expert Committee (FAO/WHO, 1969) pimaricin may be widely used in food production. While it has been permitted to be used in cheese manufacture in several countries and is being applied on an industrial scale, its application in other food processing technologies requires further investigation.

The antibiotic is manufactured by KONINKLIJKE NEDERLANDSCHE GISTEN SPIRITUSFABRIEK N. V., Delft, Holland. The preparation called *Delvocide* is an aqueous suspension containing 5% pimaricin and 0.6% NaCl.

In the experiments described herein the change during storage of the viable cell count and pimaricin activity in cream cottage cheese, prepared with *Delvocide*-containing brine at the HUNGARIAN INSTITUTE FOR DAIRYING

and packaged in plastic containers, was studied. Further the resistance to pimaricin and the lactose degrading capacity of yeast strains, isolated from spoiled cream cottage cheese at the HUNGARIAN INSTITUTE FOR DAIRYNIG and probably responsible for their spoilage, was investigated.

1. Material and methods

1.1. The test material

The cream cottage cheese prepared at the HUNGARIAN INSTITUTE FOR DAIRYING and the brine (salted whey) used in processing served as test materials. The cheese was packed in *Propathene* GWM 101 type polypropylene containers. The size of cheese and the volume ratio cheese/brine were about $12 \times 12 \times 10$ cm, and 3 : 1, resp.

The equilibrium relative humidity as measured by the crystal liquefaction method (VAS & CSONTOS, 1956) in both the cheese blocks and the brine was found to be between 93 and 96%. Further analytical data of the brine:

refractive index	20.7%,
residue after evaporation	19.7%,
acid content titrated with 0.1 *N* NaOH and expressed as lactic acid	1.2%,
NaCl (according to *Mohr*)	4.3%,
pH	4.4%.

1.2. Viable cell count determination

Surface plate count technique was used to determine viable cell counts. Universal nutrient agar* or malt nutrient agar plates were used. The preliminary experiments have shown both nutrient media to be equally suitable for yeast propagation and the number of colonies was identical. However, the universal nutrient agar seemed more suitable for the total viable cell count determinations, therefore this was used in serial tests.

Microscopic slides prepared from the colonies have shown that the bacterial colonies formed on the plates can clearly be distinguished from the yeast colonies on the basis of their morphological properties. Thus it was possible to determine the total viable aerobic cell count as well as the number of yeast cells.

* Composition of the universal medium: 200 ml sweet whey, 100 ml (1 : 1) yeast extract, 4 g meat extract, 2 g pepton, 10 g glucose, 20 g agar-agar and 700 ml water. The medium was adjusted to pH 7.2 and sterilized under 2 at gauge pressure. The pH was reduced during sterilization to 6.0–6.8.

In determining the viable cell count 0.1 ml was streaked on the plate surface in triplicate tests at each dilution level. Incubation of the plates was carried out at 30 °C and counting was done on the fourth day.

1.3. Determination of pimaricin concentration by measurement of the activity on agar plates

The fungistatic activity in pimaricin and *Delvocide* solutions and in the cheese was established by the well known agar-diffusion method. *Saccharomyces cerevisiae* var. *ellipsoideus* T_{22} strain was used as test organism. From the yeast cells grown on a malt slant and scraped off after 24-hour cultivation a dense suspension was prepared in sterile water to show 15% light transmission when using the yellow colour filter in the *Jouan* biophotometer. 0.1 ml of this suspension was smeared over the surface of malt agar slants. After 30 min desiccation 6 sterile metal cylinders in even distribution were placed on the surface of the plates and 0.1-ml portions of the antibiotic-containing test samples were introduced through these cylinders. To check the fungistatic activity in the brines and other components of the cheese samples not containing *Delvocide* and stored for the same time as the test samples were exposed to the agar-diffusion activity measurement. The samples were incubated at 30°C for four days and the inhibition zones were evaluated subsequently.

The brine was analysed directly. To determine the distribution in the cheese, slices of predetermined dimensions were cut from the cheese block with a sharp knife, these were then suspended in equal quantities of water and used for activity measurement.

In order to determine the correlation between inhibition zones and antibiotic concentration a dilution series was prepared with pure pimaricin* and used for calibration.

A linear correlation, shown in Fig. 1, was found between the logarithm of pimaricin concentration and the diameter of the inhibition zone.

The minimum effective pimaricin concentration to give an inhibition zone with the test strain was found to be at 5.5 $\mu g\ ml^{-1}$. This means that the lowest concentration limit, detectable by the method described above, was 5.5 $\mu g\ ml^{-1}$ in the brine, while in the cheese itself, assuming complete solubility of pimaricin in identical amounts of water, it was 11.0 $\mu g\ ml^{-1}$.

1.4. Pimaricin determination by spectrophotometry

Spectrophotometry was carried out in a recording spectrophotometer: *Perkin-Elmer* 137 UV.

Preliminary experiments have shown the pimaricin to be detectable in

* Produced by the AMERICAN CYANAMID Co. (Lot No. 107).

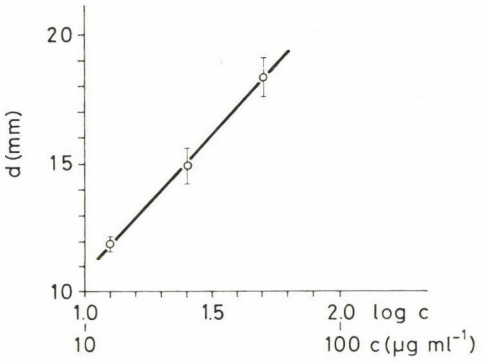


Fig. 1. Correlation between the pimaricin concentration and the diameter of the inhibition zones obtained by the agar-diffusion method. The average of 6 parallel results and standard deviations are shown. Test organism: *Saccharomyces cerevisiae* var. *ellipsoideus* T_{22}. d – diameter of the inhibition zone; c – pimaricin concentration

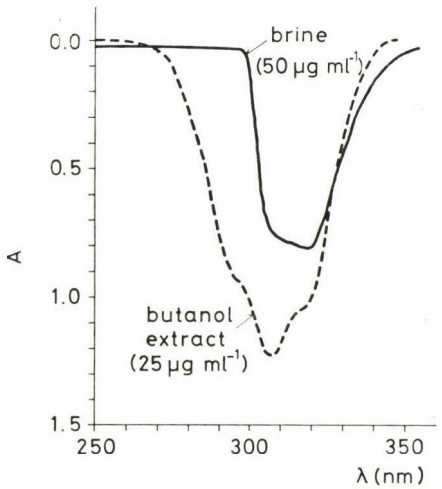


Fig. 2. Characteristic UV absorption spectrum of the pimaricin-containing brine (directly determined) and of its 1 : 1 v/v *n*-butanol extract. Cell thickness: 1 cm. *Perkin-Elmer* 137 UV recording spectrophotometer. The added pimaricin content in the brines is shown beside the spectrum (A: optical density)

the brine directly, without extraction. To analyse the cheese itself a 1 : 1 suspension in water, as used for the biological activity measurement, was shaken with an equal volume of *n*-butanol and the butanol phase was used for determination. The characteristic absorption spectra are shown in Fig. 2.

The pimaricin concentration was calculated from the optical density value found at 316 nm with a cuvette of 1 cm thickness, on the basis of the calibration curves (Figs. 3 and 4).

The calibration curves were prepared by dissolving pure pimaricin (500 μg ml^{-1}) in the brine and this stock solution was then diluted in a series

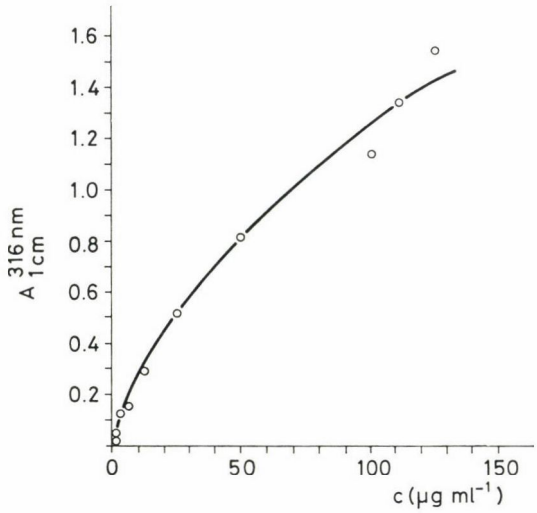


Fig. 3. Optical density value (A) as measured at 316 nm in the brine *vs.* the pimaricin concentration (c). Control solution: brine without pimaricin

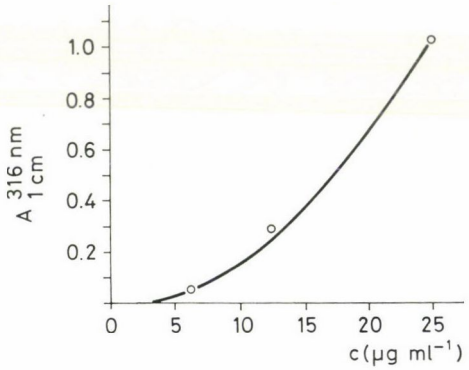


Fig. 4. Optical density value (A) at 316 nm in the 1 : 1 v/v n-butanol extract of the brine of cream cottage cheese as a function of pimaricin concentration in the brine (c). Control solution: butanol extract of the pimaricin-free brine

of solutions and these solutions or their butanol extracts were measured in the spectrophotometer.

2. Results and conclusions

2.1. Viable cell count in the brine of cream cottage cheese samples during storage

The bacterial and yeast cell counts as a function of storage time and initial *Delvocide* concentration in the brine of cheese samples stored at 10–14 °C are shown in Fig. 5.

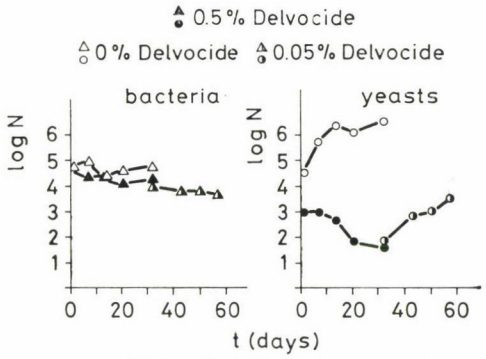


Fig. 5. Bacterial and yeast counts (N) in the brine of cream cottage cheese as a function of storage period (t) and initial *Delvocide* concentration. Storage temperature: 10–14°C

It can be seen in the figure that the bacterial counts in the *Delvocide*-containing and in the control samples did not differ substantially and changed only slightly during the test period. Probably the temperature was too low for the lactic acid bacteria to multiply. The yeast cell count in the brine containing 0.5% *Delvocide* (250 μg ml^{-1} pimaricin) was lower by 1.5 orders of magnitude than that in the control already at the initial stage of storage. With advancing storage time the cell count in the control samples increased while in the brine of high *Delvocide* concentration it decreased steadily. During the nearly one-month storage period gas formation or swelling was not observed in the control samples either. The samples of 0.05% initial *Delvocide* concentration (25 μg ml^{-1} pimaricin) were tested only after one month of storage. At the beginning of the second month the yeast count in these samples was about 5 orders of magnitude lower than in the control samples, however, during the following month a gradual increase was observed.

These observations seemed to support those of WHITEHILL (1956) who found 10 μg ml^{-1} pimaricin of fungicidal effect in cottage cheese, stored at 2.5°C. SHIRK & CLARK (1963) observed also the fungicidal effect of pimaricin at a concentration of 10 μg ml^{-1} in orange juice.

2.2. Pimaricin concentration in the brine and in the outer layer of the cheese

The results obtained in measuring the biological activity in cheese samples during storage, manufactured with brines initially containing 0.5% *Delvocide* (250 μg ml^{-1} pimaricin) and the results of spectrophotometric measurements are shown in Table 1.

The change in the pimaricin concentration in the brine is illustrated in Fig. 6.

Given the knowledge of the amount of brine in the package and the total volume and weight of the outer layer peeled off the cheese block, an assay was

Table 1

Pimaricin determination in cheese samples prepared with brine containing 0.5% Delvocide as a function of time (storage temperature: 12–14°C)

Storage period (days)	Pimaricin concentration ($\mu g\ ml^{-1}$)			
	in the brine		in the outer layer of the cheese	
	B	S	B	S
1	224	—	0–5 mm: 26	—
8	91	80	0–5 mm: 24	0–5 mm: 70
15	117	98	0–2 mm: 46	0–2 mm: 32
			2–4 mm: 30	2–4 mm: 38
			4–6 mm: < 11	4–6 mm: 22
				6–8 mm: 18
22	40	104	0–2 mm: 18	0–2 mm: 22
				2–4 mm: 20
				4–6 mm: < 11
33	73	67	0–2 mm: 55	0–2 mm: 98
			2–4 mm: 25	2–4 mm: 88
			4–6 mm: 20	4–6 mm: 40
			6–8 mm: < 11	6–8 mm: < 11

— not determined
B = activity determined by the agar-diffusion method
S = analysis by spectrophotometry

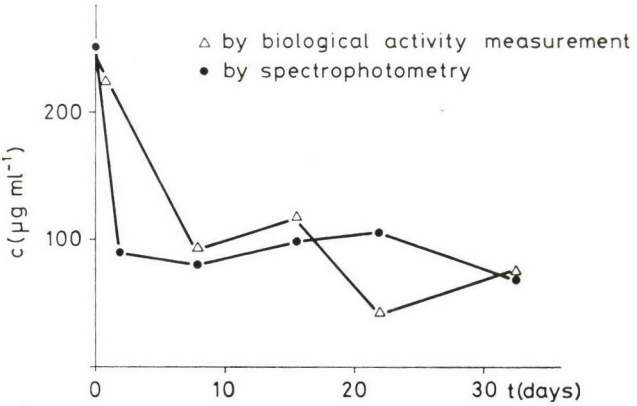


Fig. 6. Change in the pimaricin concentration of the brine (c) as a function of the storage period (t) of cream cottage cheese. Storage temperature: 10–14°C

made to determine the total pimaricin content of the whole cheese and brine in the container at the time of analysis. These results are summarized in Table 2 and illustrated in Fig. 7.

Table 2

Total pimaricin content as a function of time in cheese samples prepared with brine containing 0.5% Delvocide

(Total pimaricin content added at the beginning of the storage period: 16 250 μg)

Storage period (days)	Pimaricin content (μg)					
	in the brine		in the cheese		total	
	B	S	B	S	B	S
1	14 500	—	2 580	—	17 080	—
8	5 920	5 200	2 310	6 860	6 230	12 060
15	7 610	6 370	3 720	4 445	11 330	10 815
22	2 600	6 760	970	1 997	3 570	8 957
33	4.720	4 360	4 638	10 120	9 358	14 480

— not determined
B = activity measurement by the agar-diffusion technique
S = analysis by spectrophotometry

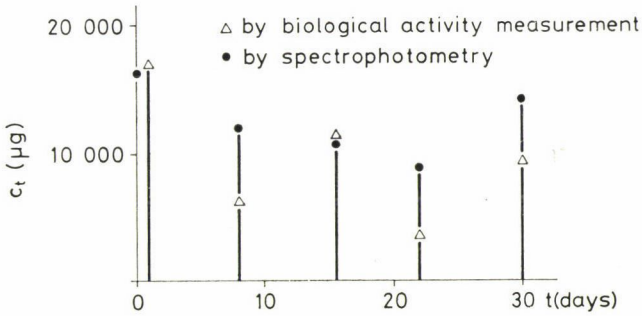


Fig. 7. Change of the pimaricin content of brine of 0.5% added initial pimaricin content as a function of the storage period (t). [Total of pimaricin content in the brine and in the cheese (c_t)]. Storage temperature: 10–14°C

The samples containing initially 0.05% added *Delvocide* (25 μg ml^{-1} pimaricin) were tested the first time on the 33rd day of storage. At this time pimaricin activity could not be demonstrated by microbiological methods either in the brine or in the cheese, thus the active pimaricin content of the brine was below 5.5 μg ml^{-1} and that of the cheese below 11.0 μg ml^{-1}. The results obtained by spectrophotometry were similar.

In spite of the deviations in the results the experiments permit of concluding that during 6 weeks of storage pimaricin penetrated the cheese block only to 1 cm depth, while only half of the initial pimaricin content could be demonstrated in the sample. However, these results are valid only under the storage conditions applied in this study and for the size of cheese as used.

CLARK and co-workers (1964) investigated the stability of pimaricin in solutions initially containing 16 μg ml^{-1} pimaricin, 3% pepton or 1% ascorbic acid and adjusted to pH 4.5. After six weeks of storage in the dark at room temperature they found a reduction of 85%, and at 2.5 °C a decrease of 50% in pimaricin activity. The stability curves established in these experiments have shown also a rapid reduction in the first stage of storage while in later stages the decrease slowed down.

According to SHIRK and CLARK (1960) the aqueous solution of 16 μg ml^{-1} initial pimaricin concentration lost approximetely 50% of its activity during 2 months of storage in the dark at room temperature and about 25% at refrigerator temperature.

SHIRK and co-workers (1964) studied the fungicidal effect of pimaricin at 10 μg ml^{-1} concentration in orange soda (6% orange juice) and observed a reduction of activity below 1 μg ml^{-1} during 6 weeks.

2.3. Lactose degrading capacity and pimaricin sensitivity of yeasts isolated from cream cottage cheese

None of the 10 yeast strains, isolated at the HUNGARIAN INSTITUTE FOR DAIRYING multiplied during the 2 weeks storage period at 30°C in a medium containing whey, lactose and 9% NaCl. Glucose was not detectable in the nutrient medium with *Glucotest* indicator paper.

The pimaricin sensitivity of these strains in comparison to that of the *Tokaj* 22 (T_{22}) strain is given in Table 3.

It can be seen in the table that the majority of yeast strains isolated from cream cottage cheese are slightly more resistant to pimaricin than *Tokaj* 22, used as test organism. However, the difference in the pimaricin sensitivity is not substantial. It amounts to only 25% with strain No. 6, appearing most resistant. Taking into account the logarithmic correlation between the zone diameter and pimaricin concentration, the most resistant cheese yeast requires double of the minimum effective pimaricin concentration of 5.5 μg ml^{-1}, as obtained for the test organism.

For the sake of comparison we mention the results obtained by AYRES and co-workers (1964) who, using the same agar-plate activity measurement technique, found 5 μg ml^{-1} the minimum inhibitory concentration in the case of *Saccharomyces cerevisiae* ATCC 9763 strain and 2.5 μg ml^{-1} for the strain marked ATCC 4109. A *Debaryomyces* strain, isolated from *Italian* salami, proved to be the most resistant with a minimum concentration of 20 μg ml^{-1}.

On the basis of the results obtained in these experiments and found in the literature pimaricin seems to be a promising fungicide in cheese manufacture.

Table 3

Pimaricin sensitivity of the yeast isolated from cream cottage cheese related to the sensitivity of Saccharomyces cerevisiae var. ellipsoideus T_{22} test organism

Yeast strain	Diameter of the inhibition zones as percentage of the zone diameter of the test organism (strain T_{22})
Saccharomyces cerevisiae var. *ellipsoideus* T_{22}	100.0
Yeast from cottage cheese:	
1.	85.3
2.	94.2
3.	97.5
4.	80.5
5.	99.5
6.	77.2
7.	107.6
8.	101.0
9.	75.4
10.	101.0

*

For raising the problem and for valuable advice given in the course of the study thanks are due to Dr. G. Szabó, Head of Department, HUNGARIAN INSTITUTE FOR DAIRYING, Mosonmagyaróvár and to the late Professor G. Pulay, KESZTHELY UNIVERSITY OF AGRICULTURAL SCIENCES, DEPARTMENT FOR DAIRYING AND MICROBIOLOGY, Mosonmagyaróvár. Thanks are due for their co-operation in the laboratory work to Miss J. Bencze-Bőcs, Mrs. A. Halmágyi, Dr. B. Kálmán and Mrs. Zs. Kothencz.

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DETERMINATION OF THE TOTAL RED AND YELLOW PIGMENT CONTENT OF SEASONING PAPRIKA WITHOUT CHROMATOGRAPHY

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Experiments were carried out to determine photometrically the pigment concentration of model solutions containing capsanthin, capsorubin, β-carotene, zeaxanthin, cryptoxanthin and lutein dissolved in benzene.

An equation is given which was used to determine the total content in capsanthin and capsorubin, giving the red colour of seasoning paprika, and the yellow pigments of β-carotene, cryptoxanthin, zeaxanthin and lutein, by measuring the extinction of the above mixture solution. The equation obtained for the model solution was applied with suitable modifications to the benzene extract of the milled paprika product. The total red and total yellow pigment content of milled seasoning paprika was determined by measuring the extinction of the benzene extract at 455 and 505 nm wave-lenghts.

The colour of milled seasoning paprika is decisively influenced by the quantitative ratio of its pigment components. Therefore, besides the total pigment quantity, it is useful to obtain information concerning the quantity of the different components. This is of special importance for determining the changes during the ripening, after-ripening and storage of paprika.

From the methods for determining the pigment content of milled seasoning paprika BENEDEK's (1958) method, as well as its modification by ANDRÉ (1973) is rapid and simple, but these methods characterize the pigment content only by an index-number proportional to the total pigment content. The thin-layer chromatographic method (TLC) elaborated by VINKLER and coworkers (1972) is suitable for determining the total red and yellow pigment quantity as well as the capsanthin and capsorubin quantity separately, the quantity of β-carotene + cryptoxanthin and zeaxanthin + lutein, resp., in pairs of these components. This method is, however, very tedious and time-consuming in the case of the numerous determinations in practice.

Thus it seemed necessary to elaborate a method of determining the pigment quantity, which, representing a transition between the above method would yield more detailed information than BENEDEK's method (1958) and, though not so rich in informations as the chromatographic method, it would be simpler and faster. Our aim was to elaborate such a method to determine the total red pigment content, consisting of capsanthin and capsorubin, and the yellow pigment content, consisting of β-carotene, cryptoxanthin, zeaxanthin and lutein, of seasoning paprika.

Our method is based on the following facts:

1. Comparing the molar extinction coefficients of the pigment components of seasoning paprika as a function of wave-length (Table 1) in benzene

Table 1

Molar extinction coefficients of the benzene solutions of capsanthin, capsorubin, β-carotene, cryptoxanthin, zeaxanthin and lutein

λ nm	Capsanthin $\varepsilon \cdot 10^{-4}$	Capsorubin $\varepsilon \cdot 10^{-4}$	β-carotene $\varepsilon \cdot 10^{-4}$	Zeaxanthin $\varepsilon \cdot 10^{-4}$	Cryptoxanthin $\varepsilon \cdot 10^{-4}$	Lutein $\varepsilon \cdot 10^{-4}$
400	2.21	1.21	2.71	3.06	2.87	3.50
405	2.60	1.49	3.39	3.75	3.54	4.18
410	3.06	1.83	4.04	4.35	4.10	4.55
415	3.45	2.28	4.67	4.92	4.66	5.16
420	4.05	2.95	5.41	5.69	5.34	5.92
425	4.60	3.64	6.46	6.68	6.26	7.54
430	5.20	4.14	7.53	7.76	7.31	8.34
435	5.97	4.58	8.33	8.60	8.15	8.37
440	6.71	5.35	8.77	8.96	8.58	8.38
445	7.44	6.56	9.23	9.26	8.90	9.34
450	8.09	7.85	10.02	10.11	9.66	11.13
455	8.81	8.63	11.22	11.38	10.82	12.46
460	9.30	8.86	12.31	12.36	11.89	12.31
465	9.76	8.96	12.60	12.44	12.09	10.66
470	10.20	9.62	11.86	11.59	11.44	9.01
475	10.66	10.91	10.79	10.47	10.35	8.72
480	11.09	12.34	10.16	9.93	9.73	9.78
485	11.28	13.09	10.16	10.22	9.89	11.05
490	11.04	13.06	10.57	10.77	10.35	10.91
495	10.57	12.08	10.58	10.81	10.49	8.78
500	9.82	10.43	9.54	9.73	9.59	5.96
502	9.62	9.62	8.56	8.76	8.68	4.73
505	9.45	9.45	7.58	7.79	7.77	3.51
510	9.22	9.68	5.32	5.23	5.56	1.87
515	8.91	11.00	3.30	3.20	3.52	0.97
520	8.34	12.00	1.81	1.79	2.02	0.50
525	7.23	11.30	0.90	0.98	1.12	0.26
530	5.94	8.77	0.37	0.42	0.46	0.16
535	4.57	5.56	0.18	0.24	0.23	0.10
540	3.33	3.25	0.03	0.16	0.15	0.04

solution it can be seen that at certain wave-lengths the mixed benzene solution containing the six pigments can be considered as a system of two components, namely a yellow pigment giving a mean extinction coefficient and a red pigment with another mean extinction coefficient. At these wave-lengths the molar extinction coefficients of β-carotene, zeaxanthin, cryptoxanthin and lutein are identical or only slightly different, and at the same time the extinction coefficients of capsanthin and capsorubin are also nearly the same. These wave-lengths are: 455, 475, 502, 505 and 540 nm (Table 1).

2. According to the literature (HAMPEL, 1962), the following simultaneous equations hold for the extinction coefficients of two-component mixed solutions, if certain conditions are fulfilled:

$$\begin{aligned} E(\lambda_1) &= \varepsilon_1(\lambda_1)c_1 + \varepsilon_2(\lambda_1)c_2 \\ E(\lambda_2) &= \varepsilon_1(\lambda_2)c_1 + \varepsilon_2(\lambda_2)c_2 \end{aligned} \tag{1}$$

In these equations $E(\lambda)$ denotes the extinction of the mixed solution for 1 cm layer thickness, $\varepsilon_i(\lambda_i)$ the molar extinction coefficients of the components, and c_i their concentration in M. In the system investigated by us, ε_1 and ε_2 are the average of the extinction coefficients of red and yellow pigments, resp., while c_1 and c_2 are the concentrations of the red and yellow pigments.

The conditions necessary for the validity of the simultaneous equations are as follows:

a) In a given concentration and wave-length range, *Beer*'s law must hold.

b) The extinction of the mixed solution in the solvent must be exactly equal to the sum of the extinctions of the single components.

If the simultaneous equations are valid, the concentration of the components can be determined by solving the equations.

In the first phase of our investigations we wished to obtain an answer to the question, whether the conditions necessary for the application of the equations are fulfilled in the case of benzene solutions of the six most important pigment components of seasoning paprika.

1. Materials and methods

1.1. Materials

Part of our investigations was made on model solutions. The measure ments were carried out on benzene solutions of specially purified capsanthin capsorubin, β-carotene, cryptoxanthin, zeaxanthin and lutein, supplied by the INSTITUTE OF CHEMISTRY, MEDICAL UNIVERSITY, Pécs, as well as on the benzene solution of the mixture of these six pigments. For the determination o

the total red and yellow pigment content of the seasoning paprika, we employed the benzene pigment extract used in the method modified by ANDRÉ (1973) for the total pigment concentration.

The concentrations of the model solutions were chosen to cover the possible concentrations of the corresponding pigments in the benzene pigment extract used by ANDRÉ (1973). Accordingly, the validity of *Beer*'s law was studied in the concentration range 10^{-7} to 10^{-5} M for the benzene solutions of pigments. The concentrations of the pigments in the mixture solutions are shown in Table 2.

Table 2

Composition of the model solutions

Pigment	Concentration of pigment in the mixture solutions, M		
	A	B	C
Capsanthin	$2.76 \cdot 10^{-6}$	$1.380 \cdot 10^{-6}$	$0.690 \cdot 10^{-6}$
Capsorubin	$2.98 \cdot 10^{-7}$	$1.490 \cdot 10^{-7}$	$0.745 \cdot 10^{-7}$
β-carotene	$5.96 \cdot 10^{-7}$	$2.980 \cdot 10^{-7}$	$1.490 \cdot 10^{-7}$
Zeaxanthin	$4.39 \cdot 10^{-7}$	$2.195 \cdot 10^{-7}$	$1.097 \cdot 10^{-7}$
Cryptoxanthin	$2.35 \cdot 10^{-7}$	$1.175 \cdot 10^{-7}$	$0.587 \cdot 10^{-7}$
Lutein	$4.41 \cdot 10^{-7}$	$2.205 \cdot 10^{-7}$	$1.102 \cdot 10^{-7}$
Total red	$3.058 \cdot 10^{-6}$	$1.529 \cdot 10^{-6}$	$0.765 \cdot 10^{-6}$
Total yellow	$1.711 \cdot 10^{-6}$	$0.855 \cdot 10^{-6}$	$0.427 \cdot 10^{-6}$
Total pigment	$4.769 \cdot 10^{-6}$	$2.384 \cdot 10^{-6}$	$1.192 \cdot 10^{-6}$

1.2. Method of measurement

The benzene pigment extract of seasoning paprika was prepared as described by ANDRÉ (1973). The solution was left to settle, then the extinction of the supernatant was measured in a 1 cm cuvette at two different wavelengths as described in para. 2.4.

The extinctions of both the model solutions and the benzene extract were measured on a grating spectrophotometer *Optica* (Milan) Type CF4 DR equipped with a recording device. The resolution of the spectrophotometer was $\delta\lambda < 0.2$ nm within an error of $\pm$ 0.15%. The dimensions of the cuvette were chosen to give extinctions between 0.2 and 0.8. The solutions were kept in a cooled medium during the measurements.

2. Results

2.1. Determination of molar extinction coefficients

In order to develop the method it was necessary to know with high precision the molar extinction coefficient of the components. Therefore we determined the molar extinction coefficients of the benzene solutions of the pigment components of paprika by measurements made in the 400–540 nm wave-length range at 5 nm intervals. The results are listed in Table 1.

The system, consisting of six pigments, was examined, whether it could be considered as a two-component one at the wave-lengths mentioned in the introduction. The calculations are very lengthy, this is why we publish them just for the two wave-lengths employed during our investigations. The simultaneous equations (1) in the case of a system of six components and two wave-lengths are as follows:

$$\begin{aligned} E(\lambda_1) &= c_1\varepsilon_1(\lambda_1)+c_2\varepsilon_2(\lambda_1)+c_3\varepsilon_3(\lambda_1)+c_4\varepsilon_4(\lambda_1)+c_5\varepsilon_5(\lambda_1)+c_6\varepsilon_6(\lambda_1) \\ E(\lambda_2) &= c_1\varepsilon_1(\lambda_2)+c_2\varepsilon_2(\lambda_2)+c_3\varepsilon_3(\lambda_2)+c_4\varepsilon_4(\lambda_2)+c_5\varepsilon_5(\lambda_2)+c_6\varepsilon_6(\lambda_2) \end{aligned} \qquad (2)$$

The extinctions of the three mixed solutions (the concentrations are given in Table 2) were calculated at both wave-lengths, employing the simultaneous equations (1) and (2). The results are summarized in Table 3.

Table 3

Comparison of extinctions calculated by the simultaneous equations (1) and (2)

Mixture solutions	E^1_{455}	E^2_{455}	$E^1_{455}-E^2_{455}$, %	E^1_{505}	E^2_{505}	$E^1_{505}-E^2_{505}$, %
A	0.4651	0.4659	−0.18	0.4027	0.4021	+0.16
B	0.2325	0.2329	−0.21	0.2013	0.2011	+0.15
C	0.1162	0.1165	−0.21	0.1007	0.1005	+0.15

The concentrations diminish in the direction from A to C. The extinctions calculated by using the simultaneous equations (1) were marked by E^1 while E^2 denotes the same results with equations (2). The wave-lengths are indicated by lower indices. It was shown that the maximal difference between the extinctions calculated by the different simultaneous equations was only 0.21%. This difference is lower than one can get during the measurement of the extinction. Moreover, it was proven by this result that at 455 and 505 nm wave-lengths the system of six components investigated by us can be considered as a two-component one, composed of red and yellow pigments.

2.2. Examination of the validity of Beer's law

We have examined the validity of *Beer*'s law for the benzene solutions of the six paprika pigment components separately in the concentration ranges given in para. 1.1. The measurements proved the validity of *Beer*'s law for all six pigment components in the wave-length range 400–500 nm (FEKETE *et al.*, 1975).

2.3. Investigation of the mixed benzene solution of the six pigment components

We prepared the mixed solution of the six pigment components of paprika by weighing in exactly the pigment quantities given in para. 1.1 and measured the extinction of the mixed solution referred to 1 cm layer thickness for every 5th nm in the wave-length range 400–540 nm. Then, knowing the concentrations and the molar extinction coefficients of the components, we calculated the extinctions of the mixed solutions for every 5th nm of the above wave-length range, assuming additivity of the extinction coefficients of the components. We compared the measured and calculated values of the extinction by plotting them as a function of wave-length in the same co-ordinate system (Fig. 1). It can be seen from the figure that the differences between measured and calculated values are very slight, not exceeding 2 %. Therefore the extinction of this system can be obtained with sufficient exactness by adding the extinctions of the single components.

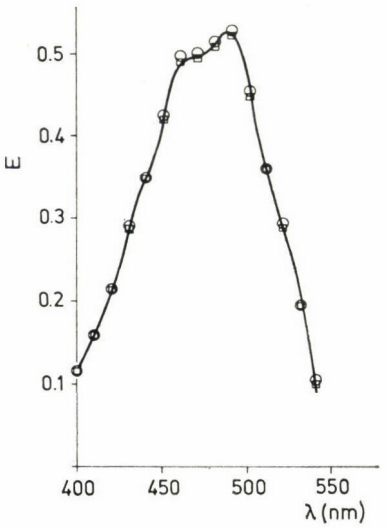

Fig. 1. Extinction curves of the benzene solution of the mixture containing capsanthin, capsorubin, β-carotene, cryptoxanthin, zeaxanthin and lutein: — □ — measured extinction curve; — ○ — calculated extinction curve

2.4. Determination of the wave-lengths most suitable for the calculation of the concentration

We had to determine the wave-lengths most suitable for the calculations because the exactness of the concentration values obtained by solving the simultaneous equations (1) is highly influenced by the choice of the wave-lengths. According to HAMPEL (1962) the most two favourable wave-lengths can be formed at the place of maximal difference between the extinction coefficients of the two components. The data necessary for determination of these wave-lengths are collected in Table 4.

Table 4

The averages and the differences of the extinction coefficients resp., as a function of the wave-lenghts

λ nm	$\varepsilon_{red} \cdot 10^3$	$\varepsilon_{yellow} \cdot 10^3$	$\Delta\varepsilon \cdot 10^3$
455	87.9	114.7	−26.8
475	106.8	100.8	+6.0
502	96.2	76.8	+19.4
505	94.5	66.5	+28.0
540	33.2	0.095	+23.7

The first column contains those wave-lengths, where the system can be considered as a two-component one. The following two columns are the averages of the extinction coefficients of red and yellow pigments, resp., while the last one contains the differences of the extinction coefficients. On the basis of Table 4, 455 and 505 nm were considered as the best wave-lengths for calculation. According to the above results, the measurements of the extinctions of the mixed pigment solutions were performed at 455 and 505 nm, in order to determine the quantities of the total red and yellow pigments.

3. Conclusions

In the case of the model solution, the calculation of concentrations was reduced to substitution into the following equations:

$$c_{\text{total red}} = \frac{E_{455} \cdot 66.5 \cdot 10^3 - E_{505} \cdot 114.7 \cdot 10^3}{-4.985 \cdot 10^9} \tag{3a}$$

$$c_{\text{total yellow}} = \frac{E_{505} \cdot 87.9 \cdot 10^3 - E_{455} \cdot 94.5 \cdot 10^3}{-4.958 \cdot 10^9} \tag{3b}$$

In these equations E denotes the extinction (optical density) of the mixture solution of 1 cm layer thickness measured at the wave-lengths denoted by the indices, and the constants $87.9 \cdot 10^3$, $94.5 \cdot 10^3$ and $114.7 \cdot 10^3$, $66.5 \cdot 10^3$ are the mean extinction coefficients of the red and yellow pigments at 455 and 505 nm, resp. Equations (3a) and (3b) give the concentrations in M units.

The results obtained with the model solution are summarized in Table 5, which shows the pigment quantities calculated from equations (3a) and (3b) as well as those weighed in. Our calculations were made in case of the three different concentrations of mixed pigments (A, B and C, see Table 1), but the relations between the pigments in all the solutions were the same (64% of red and 36% of yellow pigments). This percentage falls within the category given by VINKLER and KISZEL-RICHTER (1972) for the milled seasoning paprika (60–73% of red and 25–37% of yellow pigments), while the relations between the components within the red or yellow pigments correspond to the data of CHOLNOKY (1937). The results prove that this method gives the quantity of the total red pigment with a deviation of $\pm 2\%$ and that of the total yellow pigment with $\pm 3\%$.

Table 5

Concentrations weighed in and calculated in the model solutions

		Mixture solution		
		A	B	C
Total red pigment	c_{weighed} M	$3.058 \cdot 10^{-6}$	$1.529 \cdot 10^{-6}$	$0.765 \cdot 10^{-6}$
	$c_{\text{calculated}}$ M	$3.03 \cdot 10^{-6}$	$1.514 \cdot 10^{-6}$	$0.772 \cdot 10^{-6}$
$\frac{\Delta c}{c_{\text{weighed}}}$		−2%	−1%	+1%
Total yellow pigment	c_{weighed} M	$1.711 \cdot 10^{-6}$	$0.855 \cdot 10^{-6}$	$0.427 \cdot 10^{-6}$
	$c_{\text{calculated}}$ M	$1.750 \cdot 10^{-6}$	$0.872 \cdot 10^{-6}$	$0.440 \cdot 10^{-6}$
$\frac{\Delta c}{c_{\text{weighed}}}$		+3%	+2%	+3%

In our further work we transferred the results obtained with the model solution to the natural system, namely the benzene extract of paprika. The benzene extract of paprika contains, beside the six pigments of the model solution, also some other pigments in minor quantities. It was determined by chromatography and calculations that in benzene solutions these pigments can raise the extinction with 20% in the average at 455 nm, contrary to the 0–2% raising of the same data given at 505 nm. With respect to this circumstance, and to the rule of practice, *i.e.* that the quantity of the pigments is given in g kg^{-1} units, the relations for determination of the red and yellow pigment

quantities were modified as follows:

$$c_{\text{total red}} = \frac{(E_{455} - E_{455} \cdot 0.2) \cdot 66.5 \cdot 10^3 - E_{505} \cdot 114.7 \cdot 10^3}{-4.985 \cdot 10^9} \cdot \frac{589.2}{b} \cdot \frac{10^3}{10} \quad (4a)$$

$$c_{\text{total yellow}} = \frac{E_{505} \cdot 87.9 \cdot 10^3 - (E_{455} - E_{455} \cdot 0.2) \cdot 94.5 \cdot 10^3}{-4.985 \cdot 10^9} \cdot \frac{556.4}{b} \cdot \frac{10^3}{10} \quad (4b)$$

In these relations 0.2 is a correcting factor, while b is the quantity in g of the paprika weighed in for preparing the extract. 589.2 and 556.4 are the mean molecular weights of the red and the yellow pigments, resp.

Employing equations (4a) and (4b) we determined the total pigment content of four quality standard samples of seasoning paprika. The results can be found in Table 6.

Table 6

Calculated pigment content of milled seasoning paprika brands

Brand	Calculated pigment content, g kg^{-1}		
	Total red pigment	Total yellow pigment	Total pigment
Delicatesse quality	1.570	1.034	2.604
Sweet choice brand	1.570	0.939	2.509
Rose	1.210	0.683	1.893
Hot	1.046	0.561	1.607

The total red and yellow pigment content of the same four standard samples was determined by thin-layer chromatography, too. The results obtained with this method are shown in Table 7.

Table 7

Pigment content of milled seasoning paprika brands determined by TLC

Brand	Pigment content determined by TLC, g kg^{-1}		
	Total red pigment	Total yellow pigment	Total pigment
Delicatesse quality	1.51	0.78	2.29
Sweet choice brand	1.53	0.71	2.24
Rose	1.15	0.54	1.69
Hot	0.86	0.45	1.31

Comparison of the two methods shows that the pigment content determined by chromatography is lower, the difference of the total pigment content amounts to 13 % in the mean of the different brands. This can partly be explained by the losses in pigment resulting from chromatography.

Summing up, it can be stated that the method permits of determining the total quantity of red and yellow pigments of paprika with sufficient accuracy and much faster than by chromatography.

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COOKING STABILITY OF PROTEINS AND ESSENTIAL AMINO ACIDS IN GREEN BEAN PODS AND DRY BEAN SEEDS

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Cooking of green or dry beans increased their weight and moisture but decreased their total solids and ash content. These changes were more pronounced in the dry samples than in the green ones. Protein content on a dry weight basis did not noticeably change during cooking. Green beans had lower histidine, isoleucine and leucine, lysine and valine, but higher methionine contents than dry beans, although they contained almost the same concentrations of arginine, phenylalanine and threonine. Cooking of green beans did not markedly change the concentration of amino acids except for lysine which was pronouncedly decreased. The protein of cooked seeds contained less amino acids than that of the uncooked seeds except for threonine and valine the values of which did not markedly change during cooking.

Bean is one of the most important legumes in Egypt, where green young pods and sun dried seeds are extensively used after cooking. Dried legumes are good sources of protein, carbohydrates, minerals and a number of vitamins (BLOCK *et al.*, 1958). Drying of pea seeds reduced their moisture and increased their solids, but did not change their protein contents. However, there was an increase in histidine, isoleucine and leucine, lysine, threonine, phenylalanine, and valine, on the other hand their arginine and methionine contents were decreased (NOAMAN & HUSSEIN, 1969).

Water soluble nutrient elements and vitamins were found to be extracted and lost from the food during cooking in water (MCHENRY, 1963) and small amounts of certain nutrients might be destroyed (HARRIS & LOESECKE, 1960). Cooked green peas contained more water and less protein than the uncooked ones (HEINZ, 1962: NOAMAN & HUSSEIN, 1969; WATT & MERRILL, 1963).

Cooking of green peas increased their isoleucine, leucine, and threonine, and decreased their arginine, histidine, lysine, phenylalanine and valine contents (NOAMAN & HUSSEIN, 1969). During cooking of dried legumes, the moisture lost during drying was regained by absorption and the legumes were softened. This required a longer cooking period than for fresh legumes. Cooking of dry pea seeds decreased their arginine, histidine, lysine, and valine but increased their isoleucine and leucine, methionine and phenylalanine contents (NOAMAN & HUSSEIN, 1969). The losses of nutrients from vegetables during cooking were mostly caused by extraction into the cooking water rather than destruction (HARRIS & LOESECKE, 1960).

This investigation was carried out in order to compare the protein and essential amino acid contents in green young bean pods with those of dry bean seeds. The other object of this study was to determine the changes occurring in the protein and indispensable amino acids of these two kinds of bean after cooking in boiling water.

1. Materials and methods

Green and dry beans used in this study were obtained from the local market.

Green young bean pods were cut to pieces of 2 cm length and washed, then cooked in boiling water for thirty minutes. The cooking water was drained and the beans were immediately cooled in water.

The dry bean seeds were washed properly and cooked in boiling water for sixty minutes. The cooking water was removed and the cooked seeds were immediately cooled.

The following chemical determinations were carried out in the raw and cooked green beans as well as in the raw and cooked dry beans.

1. Total solids were determined using a vacuum oven method (HORWITZ, 1965).

2. Total nitrogen was assessed according to the semimicro-*Kjeldahl* method as described by RANKER (1927). The protein content was calculated by multiplying the values of total nitrogen by a factor of 6.25.

3. Total ash was determined using the A.O.A.C. method (HORWITZ, 1965).

4. Arginine, histidine, isoleucine and leucine, lysine, methionine, phenylalanine, threonine, and valine were qualitatively and quantitatively determined using the paper chromatographic method of BLOCK *et al.* (1958)

2. Results and conclusions

Weight, moisture, dry matter (total solids) content, ash, total nitrogen, and total protein present in the green young bean pods and in the dry bean seeds before and after cooking are represented in Table 1. The moisture content of dry bean seeds was less than that of the green beans, the dry matter was higher in the dry seeds.

The percentage of ash (on a dry weight basis) in the green pods was higher than that of the dry seeds. The protein content was almost the same. These differences in the chemical composition between green and dry beans could be due to variations in plant maturity and to the presence of hulls in the green samples.

Table 1

Average water, ash, and protein contents (%) of raw and cooked green and dry beans

Parameters	Green beans		Dry beans	
	raw	cooked	raw	cooked
Weight (g)	100	137	100	170
Moisture (%)	86.63	92.23	8.11	47.00
Dry matter (%)	13.37	7.77	91.89	53.00
Ash (%)				
a) wet weight basis	1.42	0.68	4.24	2.21
b) dry weight basis	10.62	8.75	4.62	4.17
Total nitrogen (%)				
a) wet weight basis	0.58	0.34	0.395	2.12
b) dry weight basis	4.35	4.38	4.30	4.00
Protein (%)				
a) wet weight basis	3.66	2.05	24.58	13.28
b) dry weight basis	27.50	26.45	26.80	25.06

Green and dry beans absorbed during cooking 37 and 70 parts of water, resp. for 100 parts of the food. Cooking of green beans slightly increased their moisture and decreased their dry matter content. Ash and protein values as based on dry weight of the green beans did not noticeably drop. These changes might be mainly due to the absorption of water by the plant tissues and, to a lesser extent, to the extraction of certain amounts of water-soluble nutrients from the food. The moisture content was higher in the cooked dry bean seeds than in the uncooked dry beans. The dry matter of cooked dry seeds was less than that of the uncooked seeds. The ash and protein contents as based on dry weight of the dry seeds did not change after cooking. These changes might be mainly due to the rehydration of the dry seeds and less to the extraction of certain amounts of the water soluble solids.

The data represented in Table 2 show the concentrations of the essential amino acids studied in the raw and cooked green and dry beans. Proteins of dry beans contained more histidine, isoleucine and leucine, lysine, and valine, but less methionine than those of green beans, although the two kinds of beans contained nearly identical concentrations of arginine, phenylalanine and threonine. Total essential amino acid content of the beans increased after drying. These changes might be due to the presence of hulls in the green samples, to full maturity of the dry samples, to the drying process, and the browning reaction which might have occurred during drying, further to changes in solubil-

Table 2

The essential amino acids present in the protein hydrolysate of raw and cooked green and dry beans

(Calculated as g/100 g protein)

Essential amino acids	Raw		Cooked	
	Green pods	Dry seeds	Green pods	Dry seeds
Arginine	2.07	2.14	2.15	1.50
Histidine	4.92	7.06	5.12	6.95
Isoleucine + leucine	6.99	8.03	7.12	7.50
Lysine	2.89	4.22	1.66*	3.25*
Methionine	2.54	1.63	2.87	1.36
Phenylalanine	2.72	2.80	2.83	2.00
Threonine	3.38	3.40	3.41	3.48
Valine	5.23	7.18	5.12	7.61
Total	30.74	36.46	30.28	31.65

* Significant difference as compared to the results obtained for the raw beans. (Other changes are not significant)

ity and properties of the proteins, and to the reactions of the amino acids with other biological compounds.

The amino acid composition in the cooked pods did not noticeably differ from that in the uncooked pods except for lysine which was markedly decreased after cooking. Total concentration of the amino acids assessed in the pods did not markedly change during cooking. These changes might be due to absorption of water, and to the extraction of minor amounts of some soluble nutrients as well as to the destruction of some heat labile amino acids.

The proteins of cooked dry beans contained less arginine, histidine, isoleucine and leucine, lysine, methionine and phenylalanine than those of the uncooked beans. The concentrations of threonine and valine did not noticeably change after cooking. Total concentration of all the amino acids examined in the cooked dry beans was less than that in the raw samples. These changes might be primarily due to absorption of water and secondarily to the extraction of some soluble protein as well as to the destruction of some amino acids.

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EFFECT OF THERMAL TREATMENT ON THE ESSENTIAL AMINO ACIDS OF *VICIA FABA*, L.

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Investigations were carried out to study the amino acid composition of the proteins in dry, germinated, germinated and cooked as well as baked beans (*Foul medamis*). The protein content indicated the richness of the above mentioned forms of *Vicia faba* as a protein source. All the essential amino acids were found in the protein although in different concentrations. The leucine–isoleucine mixture constituted the highest proportion, while methionine and threonine recorded the lowest concentrations. There were no remarkable changes between germinated, germinated plus cooked and baked beans in the lysine, arginine, threonine and methionine contents. Comparison between the amounts of the minimum daily requirements of essential amino acids and the amount found in 100 g of baked or cooked germinated beans emphasizes the necessity of supplementing each of them with proteins rich in methionine and threonine.

Vicia faba L. is a legume food of superior nutritive value, commonly grown and consumed in large quantities in Egypt. The most conventional local methods of preparation of beans are baking (*i. e. Foul medamis* and *Taamia*) and cooking of germinated beans (*i. e. Foul nabet*).

Generally, beans play a very important role in human diet, supplementing foods lacking certain nutrients. Nevertheless, although the proteins of legumes are of higher quality than those of cereals, beans are lacking one factor, which limits their value as a source of high quality protein. EVERSON and HECKERT (1944) demonstrated appreciable differences in the growth promoting qualities of various legumes. They stated that leguminous protein varied in biological value according to the method of preparation.

BORCHERS and ACKERSON (1950) found that Jack beans, Velvet beans and Horse beans were improved as sources of protein by autoclaving.

MCLESTER and DARBY (1952) found that whereas grains contain about 10 to 12 per cent of protein, legumes contain about 23 per cent. In the dry state, their protein content is even higher than that of meat, regardless of the protein quality. MASSOUD (1959) reported that baked beans (*Foul medamis*) contained about 35 per cent of crude protein calculated on a dry weight basis.

The total amino acid composition of Horse beans was determined by several workers, *e. g.* EVERSON and HECKERT (1944), BORCHERS and ACKERSON (1950), MAHON and COMMON (1950), KHAN and BAKER (1957) and MASSOUD (1959). Eighteen amino acids were found in Horse bean protein.

On the other hand, thermal treatment of Horse beans has a destructive effect on certain amino acids (MASSOUD, 1959). TAIRA and co-workers (1965) reported that processing of soybean had a destructive effect on cystine, lysine and arginine, as well as on methionine.

This investigation was carried out to clarify the effect of baking and cooking of germinated beans in the conventional Egyptian method on the essential amino acid composition.

1. Materials and methods

First quality market Horse beans procured from the Asyut Governorate were used in this study. Beans obtained dry were germinated and/or cooked in the laboratory according to the conventional method previously described by MASSOUD (1959). Moisture and crude protein contents were determined according to the official methods of A.O.A.C. (1960). Essential amino acids were determined qualitatively and quantitatively by paper chromatography. The method applied herein is essentially the same as that described by BLOCK and co-workers (1958). Tryptophan content was determined colorimetrically using the method described by SPICES and DORRIS (1948).

2. Results and conclusions

Crude protein contents and the essential amino acid compositions of dry, germinated, germinated plus cooked and baked beans are summarized in Table 1. The protein content ranged from 25.79 to 31.50 per cent in *Foul medamis* and dehulled germinated beans, resp. The differences may be attributed to the fact that part of the proteins leached into the brine after baking. Results revealed, however, the relatively high content of protein in beans, which agrees with the findings of RUSSEL and co-workers (1946), MCLESTER and DARBY (1952), GHALI (1955) and MASSOUD (1959). Likewise, the results revealed that *Foul medamis* have a considerably higher protein content as compared with other foods of plant origin, *e. g.* bread 8.9%, rice 6.8% and potatoes 2.0% (ANON., 1952).

On the other hand, the results indicated the presence of ten essential amino acids in the protein of dry beans in available concentrations. Concentrations of the various essential amino acids were calculated as g/100 g protein on a dry weight basis. The leucine–isoleucine mixture constituted the highest proportion of the protein of beans forming 4.27 per cent. Threonine and methionine showed the lowest concentrations, *i. e.* 0.48 and 0.23 per cent in the protein of dry beans, resp. (Table 1).

Table 1

Average protein and essential amino acid contents of dry, germinated, germinated plus cooked and baked beans. (Calculated as g amino acid/100 g protein, on dry weight basis)

	Treatment of beans			
	Dry (raw)	Germinated	Cooked germinated	*Foul medamis*
Moisture (%)	8.22	58.68	70.78	71.75
Crude protein (%)	30.67	31.50	29.92	25.79
Lysine	1.43	1.38	1.46	1.55
Histidine	1.10	0.99	1.12	0.87*
Arginine	1.71	1.66	1.75	1.74
Threonine	0.48	0.47	0.50	0.58
Methionine	0.23	0.17	0.18	0.21
Valine	1.17	1.74*	1.83*	1.93*
Phenylalanine	1.10	1.19	1.25	1.45
Leucine–isoleucine mixture	4.27	5.60*	5.99*	5.62*
Tryptophan	1.42	1.65	0.87*	1.01*

* Significant differences as compared to the results obtained for the dry (raw) bean. (Other changes are not significant)

The protein of dry beans was found to be composed of the following amino acids in decreasing order of concentration: the mixture of leucine–isoleucine, arginine, lysine, tryptophan, valine, histidine, phenylalanine, threonine and methionine. The findings are in good agreement with those reported by MAHON and COMMON (1950), GHALI (1955) KHAN and BAKER (1957), and MASSOUD (1959).

On the other hand, the results revealed that the ten essential amino acids were also present in germinated, germinated plus cooked and in baked beans, although in different concentrations. Similarly to dry beans the leucine–isoleucine mixture was present in the highest, while threonine and methionine were present in the lowest concentrations in the protein of germinated, germinated plus cooked and baked beans. The remaining 6 essential amino acids, ranging between the two groups, were present in rather appreciable concentrations.

There were no remarkable changes in the lysine, arginine, threonine and methionine contents of either germinated, germinated and cooked or of baked beans. The concentrations of the leucine–isoleucine mixture and of valine increased significantly. Histidine and tryptophan contents decreased slightly in germinated and baked beans. The changes in the concentrations of other amino acids are not significant.

Such data are in general agreement with those cited in the literature by other investigators, *i. e.* MAUSSOUD (1959) and TAHA and co-workers (1972).

On the basis of the above mentioned findings of this study it may be concluded that the ingestion of 100 g of *Foul medamis* or cooked germinated beans would furnish — with the exception of methionine and threonine — a considerable part of the essential amino acids needed as minimum daily requirements according to ROSE (1949). This indicates the necessity of supplying these limiting amino acids using other protein foods known to be rich in them.

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INFLUENCE OF PRE- AND POST-IRRADIATION FACTORS ON THE ICED STORAGE LIFE OF FISH VARIETIES

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Studies conducted in our laboratory, have revealed that the 2–3-fold enhancement in the iced storage life of fishery products, obtained by radurization doses of gamma radiation, ensures that radurized fish can be transported to distant places.

Fresh Bombay duck (*Harpodon nehereus*) and shrimp (*Penaeus* sp. and *Metapenaeus affinis*) received from Veraval, 1 000 km from Bombay, irradiated in our laboratory with a dose of 250 krad, and transported back to Veraval, were found to have a storage life of 16–25 days at 0–2°C, depending on the pretreatment, as compared with control samples which were acceptable for a period of 5–10 days at 0–2°C. Also, several varieties of radurized fish (100–150 krad) transported to Calcutta, 2 000 km distant from Bombay, were acceptable for 20–25 days at 0–2°C as compared with controls which had a storage life of 5–10 days at the same temperature.

Fluctuation in temperature in the range of 0°C to ambient temperature is a common feature during commercial transportation of fresh fish in India. Very few trawlers are equipped for preliminary processing treatments such as evisceration, filleting or icing the catch in insulated or non-insulated holds. These operations are mainly carried out on the shore. The delay in cooling fish to ice temperature results in proliferation of different types of bacteria. Under the prevailing unhygienic conditions of handling, the fish is not only prone to rapid spoilage but it may also get contaminated with pathogenic bacteria. Although there are several regulations for the control of marketable quality of fresh fish, under practical conditions of the trade it often becomes difficult to maintain the sequence of cold chain from trawlers or processing plant to retailer's market. The subject of improvement in quality of fish by careful control of ecological factors has received considerable attention and has been comprehensively reviewed (Reay & Shewan, 1949; Lahiry *et al.*, 1963; Spencer & Baines, 1964; Cutting & Spencer, 1968; McLay, 1968). It has been shown in our laboratory that pre-processing conditions play an important role in the radiation processing of fishery products. Under adverse storage conditions prior to radiation processing, biochemical and microbiological deteriorative processes may set in, resulting in loss of quality. Likewise, if post-irradiation conditions are inadequate, the quality of fish rapidly deteriorates. Under controlled laboratory conditions, radiation processing is found to greatly enhance the useful storage life of fishery products. It was

therefore of interest to examine the shelf-life of radiation processed products under commercial conditions.

We have therefore carried out detailed investigations on transportation of radurized sea-foods with the collaboration of the CENTRAL INSTITUTE OF FISHERIES TECHNOLOGY, Sub-station Veraval (Gujarat State), and JADAVPUR UNIVERSITY, Calcutta.

Three types of investigations were conducted: *viz.* 1. fish obtained from a local market were irradiated and then transported to Calcutta by rail; 2. fish received in iced condition from Veraval were irradiated and dispatched back by rail to Veraval; and 3. fish irradiated in our laboratory were dispatched by rail to Calcutta; after reaching Calcutta, the containers were replenished with ice and returned by rail to our laboratory.

In the first two types of investigations, one-half of all samples were sent either to Calcutta or Veraval for analysis, while the other half were stored in our laboratory at 0–2°C and examined for quality characteristics at regular intervals. In the third type of investigation, *i. e.* the to-and-fro studies, samples were analysed only in our laboratory, during storage at 0–2°C. This paper reports the findings of pertinent studies conducted in our laboratory.

1. Materials and methods

1.1. Processing, packaging and transportation of irradiated fish

Several varieties of fish were brought in iced condition from nearby landing places. Of these, silver pomfrets (*Stromateus cinereus*), Hilsa (*Hilsa ilisha*), Seer fish (*Scomberomorous guttatus*), Croaker (*Johnius dissumieri* sp.), Horse mackerel (*Caranx* sp.) and mackerel (*Rastrelliger kanagurta*) were beheaded and eviscerated. Shrimps (*Penaeus* sp. and *Metapenaeus affinis*) were peeled and deveined; and Bombay ducks (*Harpodon nehereus*) were beheaded, eviscerated, filleted and pressed between two metal plates to form laminates. After washing and cleaning, the samples were drained free of water, packed in polyethylene bags (100–500 gauge), and subjected to a dose of 100–150 krad [1.0–1.5 kilogray (kGy)], in a ^{60}Co package irradiator [100 kCi = 3.7 peta becquerel (PBq)] at a dose rate of 270 krad h^{-1}. Radurized fish with respective controls were packed with three times their weight of ice in alternate layers in insulated boxes. Boxes dispatched by rail took 3–4 days to cover the distance of 2 000 km to Calcutta. In these studies, one-half of the samples were sent to Calcutta for analysis, while the other half were stored in our laboratory at 0–2°C for quality evaluation at regular intervals.

1.2. Packaging and radiation processing of transported fish

Fish samples (Table 1) received after 48 h from Veraval, 1 000 km distant from Bombay, were selected for processing after evaluating them according to the sensory rating scale of MIYAUCHI and co-workers (1964). Selected fish samples were beheaded, eviscerated and if necessary filleted, washed, packed in polyethylene bags (100–500 gauge), and exposed to 100–250 krad. Irradiated samples were packed in insulated boxes as described above, and dispatched to Veraval by rail. In these studies also, one-half of the samples were retained in our laboratory for analysis, while the remainder were sent to Veraval for analysis.

1.3. Transportation studies

Radurized fish samples transported from this Centre to Calcutta were returned to our laboratory after replenishment with ice. These samples which were in transit for about 7 days were stored at 0–2°C for quality assessment at regular intervals.

1.4. Assessment of quality

Radurized Bombay duck laminates were analysed at regular intervals for organoleptic score (OS). The scoring was done on a reference scale, based upon the odour and appearance of fish which was adopted from MIYAUCHI and co-workers (1964). The score of 5 was taken as a limiting score of acceptability of representative fish samples. Total bacterial count (TBC), total volatile basic nitrogen (TVBN) and trimethylamine nitrogen (TMAN) were determined according to methods described by GORE and KUMTA (1970).

1.5. Spoilage units

Spoilage units in terms of organoleptic ratings were calculated for Bombay duck laminates using the least squares method of SPENCER and BAINES (1964).

1.6. Assessment of toxin

Representative Bombay duck samples were homogenized with twice their weight of physiological saline and centrifuged for 20 min at 10 000 rpm in a *Sorvall* RC_2 refrigerated centrifuge. After incubation with 0.5% trypsin for 1 h at 37°C to enhance the potency of toxin (DUFF *et al.*, 1956) if present, the supernatant was injected intraperitoneally into a group of 5 mice, which were kept under observation for four days. Representative samples of unirradiated and irradiated Bombay duck were assayed for toxicity immediately and after a week, resp.

2. Results and conclusions

2.1. Extension in shelf-life of transported fish

Table 1 indicates the extension in refrigerated shelf-life of fish that were transported from Veraval and later irradiated in our laboratory with a dose of 250 krad. It can be seen from the table that the type of pre-processing treatment contributed significantly to the useful storage life of the fish. Results obtained at Veraval, which have been published elsewhere (VENKATARAMAN *et al.*, 1969) are similar to our findings.

Table 1

Extension in shelf-life of transported fish (dispatched from Veraval) at 0–2°C

Samples	Shelf-life (mean and standard deviation) (days)	
	Control	Irradiated (250 krad)
Shrimp		
Headless, shell-on	7 ± 0.55	20 ± 1.4
Peeled and deveined	7 ± 0.89	20 ± 0.63
Peeled, deveined, blanched	10 ± 1.4	(25 ± 1.4)
Bombay duck		
Whole	5 ± 0.63	16 ± 2.28
Headless and gutted	5 ± 0.89	20 ± 1.26

2.2. Extension of shelf-life of radurized fish transported to Calcutta

The results of experiments on shelf-life of radurized fish are shown in Table 2. Representative samples of radurized fish which were transported to Calcutta showed a 3–4-fold extension in shelf-life (BHADRA *et al.*, 1973). These findings are in close agreement with the observations made in our laboratory.

2.3. Transportation studies

To-and-fro studies involved transportation of Bombay duck laminates and mackerels to Calcutta and back to Bombay. In these studies it was found that unirradiated Bombay duck laminates were acceptable for 8 days while irradiated samples of both Bombay duck laminates and mackerels have a

Table 2

Extension in shelf-life of radurized fish stored at 0–2°C (transported to Calcutta)*

Sample	Laboratory findings at Bombay Shelf-life (mean and standard deviation) (days)	
	Unirradiated	Irradiated
1. *Fresh Bombay duck*		
(a) Fillets (2 batches)	8 ± 0.89	22 ± 2.40
(b) Laminates (3 batches)	8 ± 0.63	22 ± 0.78
(c) To-and-fro transported laminates	8 ± 1.10	20 ± 3.20
2. *Silver pomfrets*		
(a) Whole, eviscerated	6 ± 1.40	20 ± 2.28
(b) Headless	6 ± 1.80	20 ± 0.63
(c) Fillets	7 ± 0.63	22 ± 1.68
3. *Shrimps*		
Peeled and deveined	10 ± 1.09	25 ± 1.61
4. *Mackerel*		
Headless and eviscerated (3 batches)	5 ± 0.89	20 ± 1.18
5. *Mackerel*		
To-and-fro transported fish	Spoiled on 8th day after arrival from Calcutta	20 ± 1.18
6. *Hilsa*		
Headless and eviscerated	5 ± 0.71	25 ± 0.89
7. *Horse mackerel*	7 ± 1.41	20 ± 1.67
8. *Croaker* (2 batches)	5 ± 1.41	20 ± 2.61
9. *Seer fish*	5 ± 1.41	20 ± 2.76

* Mackerels were subjected to 150 krad whereas all other fish varieties were exposed to 100 krad

similar storage life of 20 days at 0–2°C. These results compare favourably with the 0–2°C storage period of control and irradiated Bombay duck laminates and mackerel obtained from local markets. These findings therefore indicate that the quality of fish during transportation in ice containers is comparable to that of fish stored at 0–2°C.

2.4. Quality evaluation of fresh and transported Bombay duck laminates

Organoleptic scores (OS) of unirradiated and irradiated Bombay duck fillets of several batches during storage at 0–2°C are shown in Fig. 1. It is evident from the figure that precipitous decline in organoleptic score was observed for unirradiated whole and filleted Bombay duck during storage for 10–12 days at 0–2°C. In contrast, the irradiated samples had consistently high organoleptic ratings of 7–8 until 15 days of storage. However, on prolonged storage, a fall in sensory score was noted.

Initial total aerobic bacterial count (TBC) ranged from 10^5 to $10^6 g^{-1}$ in unirradiated Bombay duck, reaching a maximum of 10^8–10^9 g^{-1} on the 12th day of storage. Irradiation at 250 krad reduced the initial bacterial load by 2–3 log cycles. The total aerobic bacterial count in irradiated samples increased to 10^7–10^8 g^{-1} after 20–25 days of storage at 0–2°C.

The higher total bacterial counts (TBC) in transported fish as compared with the samples from local sources, stored for similar periods at 0–2°C, may be attributed to fluctuations in temperature of the container during transit and/or a higher initial count of psychrophilic bacteria in fish obtained from Veraval.

Despite high bacterial counts in irradiated fish during prolonged storage at 0–2°C, these products were organoleptically acceptable up to 28 days storage. This is because gamma radiation is known to selectively eliminate spoilage organisms (Lewis *et al.*, 1971; Lewis *et al.*, 1973).

Figure 2 illustrates the patterns of total volatile basic nitrogen (TVBN) and trimethylamine nitrogen (TMAN) in whole and filleted Bombay duck stored at 0–2°C.

It may be noted that initial TVBN values in unirradiated and irradiated Bombay duck ranged from 18–23 mg%. After 12 days storage at 0–2°C, at which time the unirradiated samples were organoleptically unacceptable, it was found that the TVBN value for fillets was 32 mg% as compared with 43 mg% for whole fish samples. The findings indicate that the rate of spoilage is less in fillets than in whole fish. On the contrary, TVBN levels were maintained at 28–32 mg% for 23 days in irradiated Bombay duck, but on prolonged storage for 32 days at which time the samples were rejected, these values increased to 40 mg%. It can be seen from the figure that there is no significant difference in TVBN values of whole and filleted Bombay duck, during the entire period of storage at 0–2°C. This may be attributed to radiation destruction of TVBN-forming organisms. The initial TMAN levels in unirradiated fish which ranged from 0.1–1.0 mg% increased to 3–3.5 mg% after storage for 12 days at 0–2°C. In these studies also, fillets were found to exhibit lower TMAN values than whole fish. Irradiated samples of fillets as well as whole fish were found to maintain low TMAN values during the entire period of storage at 0–2°C.

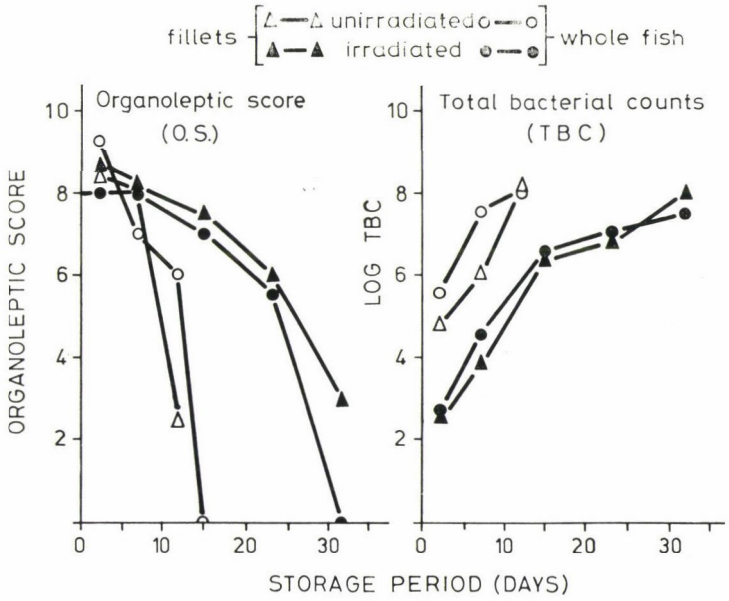

Fig. 1. Organoleptic score (OS) and total bacterial count (TBC) of irradiated and unirradiated fishery products (received from C.I.F.T., Veraval) during storage at 0–2°C. A sensory rating of 5 was taken as the borderline of acceptability. The control samples were rejected after their TBC values exceeded 10^7 g^{-1}. However, irradiated samples having TBC values up to 10^7 g^{-1} were acceptable

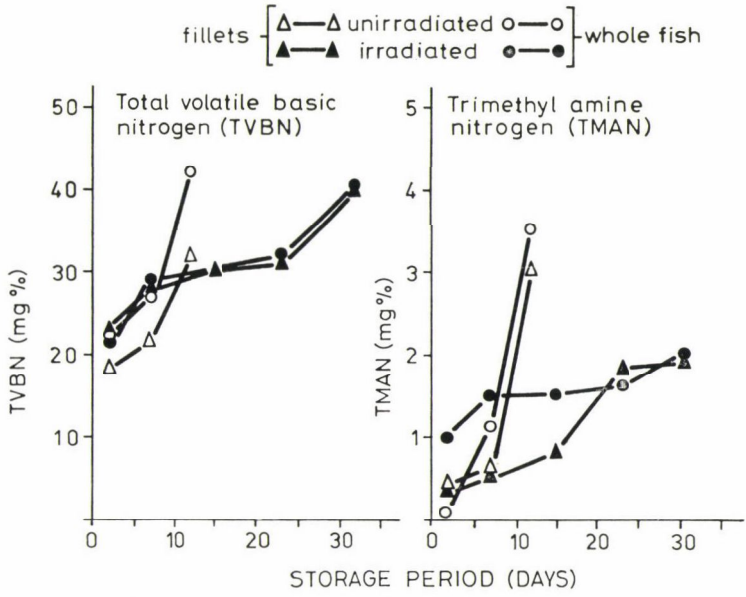

Fig. 2. Total volatile basic nitrogen (TVBN) and trimethylamine nitrogen (TMAN) content of irradiated and unirradiated fishery products (received from C.I.F.T., Veraval) during storage at 0–2°C. Both unirradiated and irradiated samples which possessed TVBN and TMAN ranging from 15 to 30 mg% and 0.2 mg%, resp., were acceptable

Table 3 illustrates the spoilage units of Bombay duck obtained from a local market and those transported from Veraval. Spoilage units in terms of organoleptic score ranged from 0.74—0.76 for unirradiated samples obtained from local markets. Upon irradiation, significant reduction in spoilage rate was noted. Thus, two-fold reduction in spoilage units was noted in samples exposed to 100 krad. On the other hand, unirradiated samples received from Veraval exhibited higher spoilage units which ranged from 1.13 to 1.58. These results indicate that deterioration in quality had occurred during transportation from Veraval to Bombay. Thus, higher spoilage units in Bombay duck transported from Veraval may be attributed to fluctuation in temperature of the container during transit, or to a higher initial count of psychrophilic bacteria in Bombay duck caught at Veraval. However, irradiation at 100 and 250 krad resulted in 2–3- and 4–5- fold reduction, resp. in spoilage units as compared with unirradiated counterparts. It is interesting to note that there is no appreciable difference in spoilage units among batches, irrespective of source and radiation dose (process treatment).

Thus, if the spoilage units of radurized products held under these conditions are predetermined, any higher spoilage units for such samples that are likely to be abused under practical conditions of refrigerated storage and distribution systems would serve as a guide-line for rejection of the samples.

Table 3

Spoilage units of locally available and transported Bombay duck stored at 0–2°C

Batch No.	Samples received from Veraval			Samples from local markets	
	Control	Irradiated		Control	Irradiated
	0 Mrad	0.1 Mrad	0.25 Mrad	0 Mrad	0.1 Mrad
1	−1.58 ± 0.13	−0.52 ± 0.12	−0.30 ± 0.11	−0.76 ± 0.07	−0.24 ± 0.037
2	−1.35 ± 0.10	−0.47 ± 0.093	−0.30 ± 0.007	−0.75 ± 0.13	−0.27 ± 0.05
3	−1.13 ± 0.26	−0.42 ± 0.10	−0.23 ± 0.04	−0.74 ± 0.086	−0.38 ± 0.28

Spoilage units per day were calculated from the storage data on OS for unirradiated and irradiated (0.1 and 0.25 Mrad) Bombay duck using the equation $U = V(1 + cT)$, where U = spoilage units per day at T °C, V = spoilage units per day at 0 °C, T = temp. (°C), and c = linear temperature response

2.5. Microbiological safety of transported and fresh Bombay duck

Bombay duck transported from Veraval, and samples collected from different parts of Bombay were examined for toxicity by mouse bioassay method. Unirradiated samples were assayed immediately, while irradiated (0.1 Mrad) samples were stored for a week before examination for toxicity. As shown in Table 4 none of the samples exhibited toxicity in the mice.

Table 4

Toxicity of Bombay duck obtained from different places in Bombay and from Veraval

Source	No. of samples toxic to mice / Total No. of samples examined	
	Unirradiated	Irradiated (0.1 Mrad)
Sassoon Dock (Bombay)	0/5	0/20
Versova (Bombay)	0/5	0/20
Grant Road fish market (Bombay)	0/5	0/20
Parel fish market (Bombay)	0/5	0/20
Batch I (Veraval)	0/5	0/10
Batch II (Veraval)	0/5	0/10
Batch III (Veraval)	0/5	0/10
Batch IV (Veraval)	0/5	0/10

Microbiological safety of radurized fishery products has received considerable attention (CANN *et al.*, 1966). Bombay duck laminates exposed to 0.1 Mrad can be stored for 15 days at 0°C as against 5–6 days for unirradiated laminates. In the present investigation, the laminates were also stored at 8–10°C with a view to assess the possible development of toxin under accelerated growth conditions assuming the existence of *C. botulinum* species in Bombay duck. The mouse lethal tests with unirradiated as well as irradiated laminates revealed the absence of any toxicity.

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RADIATION PRESERVATION OF BOMBAY DUCK (*HARPODON NEHEREUS*) LAMINATES

S. N. DOKE, U. S. KUMTA and N. F. LEWIS

(Received July 18, 1975)

A method for preparing Bombay duck laminates has been described. A radiation dose of 0.25 Mrad is found to extend the shelf-life of Bombay duck laminates up to 20–22 days at 0–2°C as compared with unirradiated samples which spoiled within 5 days.

Total volatile basic nitrogen is a good index of spoilage for unirradiated samples and samples irradiated up to 0.25 Mrad, while at higher doses of radiation it is not a good index of spoilage. In contrast, trimethylamine is a good index of spoilage for both unirradiated and irradiated Bombay duck laminates.

Bombay duck, a locally available fish, is not readily amenable to conventional methods of preservation like canning or freezing due to its extremely high moisture content and labile structural proteins which lead to tissue disintegration during processing. Sun-drying is the only method hither to adopted for conservation of this food commodity, but this product also, is of a poor quality. Studies (SAWANT *et al.*, 1967) in our laboratory have indicated that radurization of Bombay duck extends the shelf-life of this fish in "as-is" condition for 3 weeks at refrigerated temperature (8–10°C). The main disadvantage is that Bombay duck fillets exude a large amount of drip during irradiation and subsequent storage which, however, can be minimized by pre-dip treatments in NaCl and polyphosphate solutions (GORE & KUMTA, 1970).

Recent studies in this laboratory have revealed that Bombay duck laminates, prepared by compressing fillets to release surplus tissue fluid, need not be subjected to a pre-dip in salt solutions, prior to radurization.

1. Materials and methods

1.1. Preparation of fillets and laminates

Bombay duck (*Harpodon nehereus*), freshly caught on the shores around Bombay, were brought to the laboratory in ice. Fish were washed thoroughly before and after evisceration, and cut into fillets. After removal of the adhering water by folding the fillets between sheets of filter paper, the fish fillets

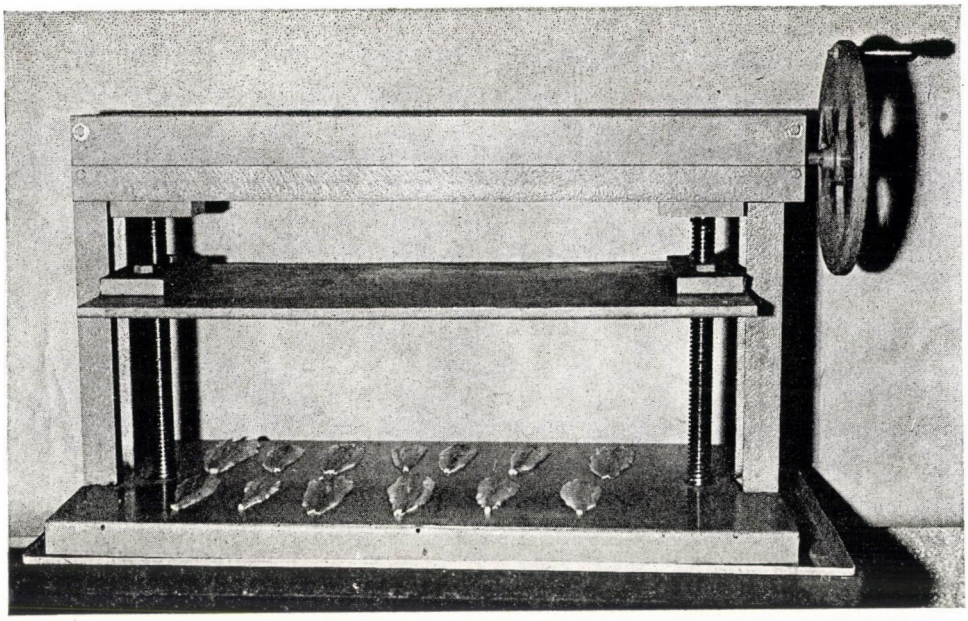

Fig. 1. Machine used for preparing Bombay duck laminates

were pressed between two metal plates for about 5 min to remove surplus tissue fluid. The laminates thus prepared were packed in polythene bags (500 gauge) and stored in ice until used. Fig. 1 depicts the machine used for preparing Bombay duck laminates.

1.2. Irradiation

The fish laminates were irradiated at 0.1, 0.25 and 0.5 Mrad [1.0, 2.5 and 5.0 kilogray (kGy)] at a dose rate of 0.27 Mrad h^{-1} in a ^{60}Co package irradiator [100 kCi = 3.7 peta becquerel (PBq)]. The temperature during irradiation was maintained at 8–10°C. Unirradiated samples served as controls. Both irradiated and unirradiated samples were stored at 0–2°C.

1.3. Quality evaluation

At regular intervals during storage, fish samples were analysed for organoleptic attributes by five trained panelists, using *Miyauchi*'s sensory rating scale (MIYAUCHI *et al.*, 1964). A sensory rating of 5 was taken as the borderline of acceptability.

Total bacterial count (TBC), trimethyl amine nitrogen value (TMAN) and total volatile basic nitrogen value (TVBN) were assessed according to methods described earlier (SAWANT *et al.*, 1967).

2. Results and conclusions

Evidence obtained from the present investigation revealed that storage at 0–2° C limits the shelf-life of unirradiated Bombay duck laminates to 4–5 days. In contrast, irradiated fish samples show dose dependent extension in the shelf-life. Exposure of the laminates to 0.1 and 0.25 Mrad did not alter the organoleptic attributes of the product significantly, while irradiation at 0.5 Mrad gave rise to burnt-type odours which, however, diminished during subsequent storage. These findings are in agreement with the work of SAWANT and co-workers (1967) on Bombay duck fillets. Fig. 2 incorporates data on organoleptic score of irradiated and unirradiated Bombay duck laminates. It can be seen from this figure that the storage life of laminates exposed to 0.5 Mrad is about 40 days but these samples exhibit an unpleasant smell thereby reducing their acceptability. Irradiation of laminates at a dose level of 0.1 and 0.25 Mrad extended the shelf-life to 15 and 20 days, resp., as compared with controls which were rejected after five days. From Fig. 2 it is evident that 0.25 Mrad is the optimum dose for extending the refrigerated life of Bombay duck laminates.

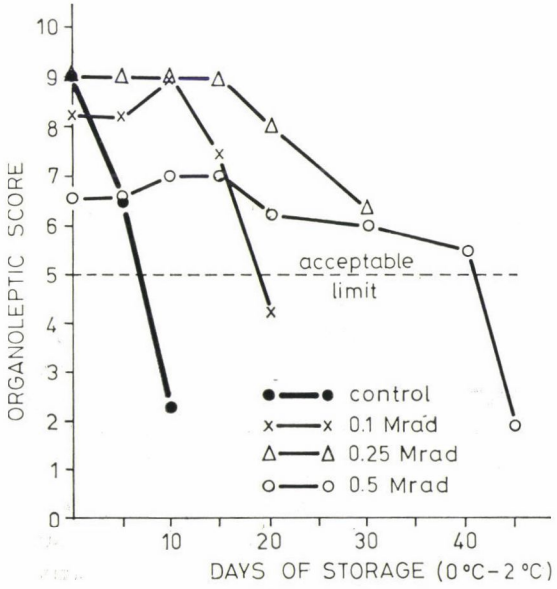

Fig. 2. Organoleptic scores of irradiated and unirradiated Bombay duck laminates stored at 0–2°C

2.1. Chemical indices of freshness

Figures 3 and 4 give the distribution of TVBN and TMAN values of irradiated Bombay duck laminates stored at 0–2°C.

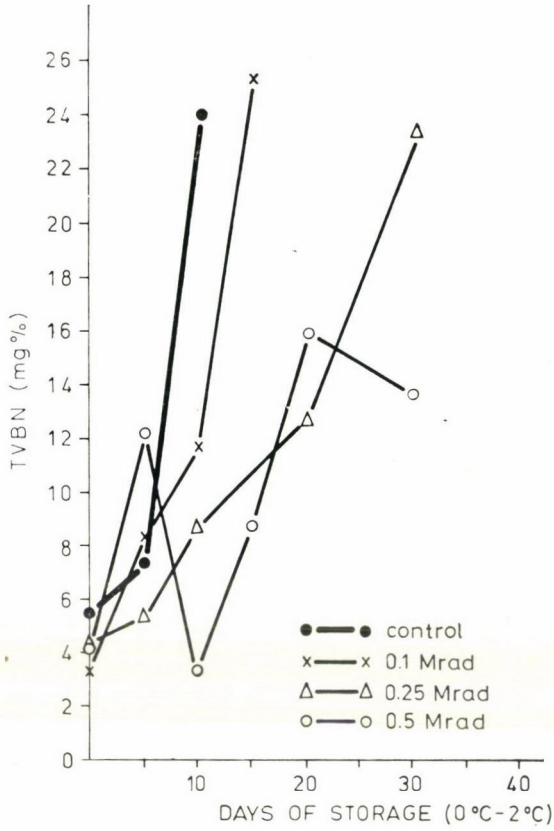

Fig. 3. TVBN values of irradiated and unirradiated Bombay duck laminates stored at 0–2°C. Each point represents the average of three independent experiments

A linear relationship was observed between TVBN content and storage period in unirradiated samples, and samples subjected to doses of 0.1 and 0.25 Mrad. However, this trend is not observed in samples irradiated at 0.5 Mrad. This indicates that TVBN is a good index of spoilage for unirradiated samples and samples irradiated up to 0.25 Mrad. The initial values of TVBN expressed in mg N per 100 g fish for 0.1 and 0.25 Mrad dose level was 3.9 and 4.4 which rose to 25.2 and 23.5, resp., at the end of the storage period.

As can be seen in Fig. 4, TMAN is a good index of spoilage for both unirradiated and irradiated fishery products, representative samples showing high TMAN values only after their useful storage life at 0–2°C.

2.2. Microbiological status

Table 1 indicates the microbiological status of unirradiated and irradiated Bombay duck laminates stored at 0–2°C. Irradiation of laminates at

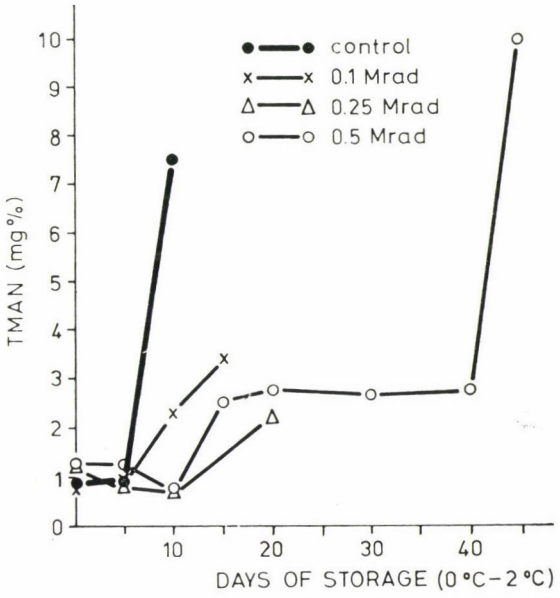

Fig. 4. TMAN values of irradiated and unirradiated Bombay duck laminates stored a 0—2° C. Each point represents the average of three indepent experiments.

Table 1

Total bacterial counts (mean and standard deviation, cells per g) of unirradiated and irradiated Bombay duck laminates stored at 0–2°C

Days of storage	Radiation dose (Mrad)			
	0	0.1	0.25	0.5
0	$(5.0 \pm 0.33) \cdot 10^5$	$(6.3 \pm 0.27) \cdot 10^4$	$(8.2 \pm 0.26) \cdot 10^3$	$(3.6 \pm 0.46) \cdot 10^2$
5	$(6.9 \pm 0.35) \cdot 10^5$	$(2.9 \pm 0.36) \cdot 10^5$	$(4.2 \pm 0.29) \cdot 10^4$	$(6.0 \pm 0.60) \cdot 10^2$
10	$(2.8 \pm 0.24) \cdot 10^8$	$(5.5 \pm 0.17) \cdot 10^7$	$(2.4 \pm 0.14) \cdot 10^6$	$(8.8 \pm 0.60) \cdot 10^3$
15	—	$(1.9 \pm 0.14) \cdot 10^8$	$(6.9 \pm 0.20) \cdot 10^7$	$(6.2 \pm 0.18) \cdot 10^6$
20	—	$(2.7 \pm 0.14) \cdot 10^8$	$(1.7 \pm 0.20) \cdot 10^8$	—
30	—	—	$(1.3 \pm 0.28) \cdot 10^8$	$(1.2 \pm 0.11) \cdot 10^7$
40	—	—	—	$(2.8 \pm 0.24) \cdot 10^8$
45	—	—	—	$(4.2 \pm 0.26) \cdot 10^8$ (spoiled)

0·1–0.25 Mrad reduced the total bacterial counts from $5 \cdot 10^5$ g^{-1} to $6.3 \cdot 10^4$ g^{-1} and $8.2 \cdot 10^3$ g^{-1} in the case of 0.1 and 0.25 Mrad samples, resp. and $3.6 \cdot \cdot 10^2$ g^{-1} in the case of 0.5 Mrad sample. The controls were found to possess high TBC values of $2.8 \cdot 10^8$ g^{-1} after 10 days, at which time they had developed putrid odours. In the case of irradiated samples TBC increased at a much slower rate than in controls, and although TBC values were in excess

of 10^8 g^{-1} after 15–20 days storage, there was no sign of putrefaction. Some sweetish odours could be detected after 3 weeks storage. This is due to the shift in microflora caused by radiation (MAVINKURVE *et al.*, 1967; LEWIS *et al.*, 1971; ALUR *et al.*, 1971). Shifts in microflora of radurized sea-foods have been reported by several researchers (ALUR *et al.*, 1971; PELROY & EKLUND, 1966; KAZANAS, 1966).

2.3. Protein content

Although the production of laminates results in a weight reduction of about 20%, it is found that most of the tissue fluid contains water with only a marginal quantity of proteinaceous material. This is indicated in Tables 2 and 3.

In conclusion it may be emphasized that the preparation of laminates from fresh Bombay duck offers an excellent method for conservation of this fish at ice temperature (0–2°C) by exposing them to a low dose of gamma radiation. Radurization of this food commodity could be thus greatly beneficial in bridging the current protein malnutrition gap in this country.

Table 2

Loss of tissue fluid of Bombay duck fillets when pressed between two metal plates (means and standard deviations)

Weight of fillets (g)	Weight of laminates (g)	Loss of weight (g)	% Loss
1. 165 ± 32.09	132 ± 16.16	33 ± 6.17	20 ± 3.68
2. 185 ± 25.09	150 ± 17.03	35 ± 9.57	19 ± 2.09
3. 135 ± 18.44	107 ± 22.43	28 ± 8.27	20.7 ± 2.0

Table 3

Protein content of Bombay duck fillets, laminates and tissue fluid (means and standard deviations)

Sample	(Protein (% of muscle)
Bombay duck fillets	10 ± 0.57
Bombay duck laminates	10.8 ± 0.46
Tissue fluid	11.6 ± 0.49

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KINETIC ANALYSIS OF PROTEIN SYNTHESIS IN FUNGI

PART II. — THE EFFECT OF COMPOSITION OF MEDIA ON GROWTH AND PROTEIN FORMATION OF *MUCOR MUCEDO*

K. ZETELAKI-HORVÁTH, K. VAS,
A. ABD EL-BAKEY MAHMOUD and E. BÉKÁSSY-MOLNÁR

(Received September 4, 1975)

Mycelial and protein yields of a *Mucor mucedo* strain (No. 224) were compared in synthetic, semi-synthetic and natural media with special regard to the time of harvest. Growth and protein formation were kinetically evaluated.

Cultures grew according to regular four-phase growth curves in synthetic glucose and in bread medium, but diauxic growth occurred when synthetic culture media with sucrose and semi-synthetic media with sucrose and glucose were used.

Protein formation in the culture proved to be growth-related in each of the media used.

The highest rates of growth and protein formation (0.200 and 0.100 h^{-1}, resp.) were attained in the first exponential phase of the culture incubated in semi-synthetic sucrose medium.

Highest mycelial yield (34 g l^{-1}) was obtained in the 60-hour culture grown in bread medium, while the maximum of the protein yield (16 g l^{-1}) was attained 6 hours earlier. The protein yields of the cultures in semi-synthetic media were about 11 g l^{-1} while in synthetic media they were below 10 g l^{-1}.

To investigate the possibilities of converting carbohydrate-containing waste products into protein-rich biomass, growth and protein production of several fungal strains were studied (ZETELAKI-HORVÁTH *et al.*, 1975; ZETELAKI-HORVÁTH & VAS, 1976). For a large-scale production of fungi the kinetic analysis of their growth and product formation can give valuable information about their behaviour during cultivation.

In the present paper the growth and protein formation of a *Mucor mucedo* strain were kinetically analysed when cultures were incubated in synthetic, semi-synthetic and in natural media.

1. Materials and methods

1.1. Microorganism

A *Mucor mucedo* strain (No. 224) was used as the test organism (from the Strain Collection of the NATIONAL RESEARCH INSTITUTE FOR VITICULTURE AND ENOLOGY, Budapest). Stock cultures were maintained on agar slants.

1.2. Nutrient media

For *inoculation* the following medium was used: yeast 5.0 g (in extract form); soluble starch 15 g; KH_2PO_4 1.0 g and $MgSO_4 \cdot 7\ H_2O$ 0.5 g adjusted to 1 000 ml with tap water; pH 5.5.

As *fermentation* media synthetic (**Nos. 1** and **3**), semi-synthetic (**Nos. 2** and **4**) and natural media (**No. 5**) were used: **No. 1**: glucose 80 g; **No. 2**: glucose 80 g; corn-steep liquor 20 g; **No. 3**: sucrose 80 g; **No. 4**: sucrose 80 g; corn-steep liquor 20 g; **No. 5**: ground bread 40 g; 400 g of dry ground bread were suspended in 9 400 ml of tap water. This was heated to 80°C, then 16 ml of conc. H_2SO_4 were added. The hydrolysis was carried out at 120°C for 60 min. After hydrolysis the pH was adjusted to 4.0 with NaOH.

Minerals were added to each of the above media as follows: $(NH_4)_2SO_4$ 10 g; $ZnSO_4$ 0.25 g; $MnSO_4$ 0.10 g; KH_2PO_4 1.0 g. The volume of each medium was made up to 1 000 ml with tap water; their pH adjusted to 4.0.

1.3. Method of cultivation

100 ml of inoculum media were inoculated with 1 ml of a spore suspension (containing 10^6 conidia per ml) and were incubated for 24 hours on a rotary shaker (rpm 330; stroke 20 mm; O_2-transfer rate in the flasks 17–19 mmole $O_2\ l^{-1}\ h^{-1}$) at a temperature of 28°C. Cultivation was carried on in 10-litre glass fermenters. The 6 000 ml of medium contained in a fermenter were inoculated with 600 ml of the above 24-hour vegetative culture. The speed of agitation was 460 rpm, and the volume of air that was bubbled through the cultures was 1.0 $l\ l^{-1}\ min^{-1}$. The oxygen transfer rate in the fermentor was 50.8 mmole $O_2\ l^{-1}\ h^{-1}$, determined by the method of COOPER and coworkers (1944).

1.4. Preparation of the mycelia

Mycelia were obtained from the fermentation broth by filtration through a Nylon cloth. The residues of the medium were removed by thorough washing in water, then the mycelia were dried at 105°C to constant weight.

1.5. Protein determination

The protein content of the mycelia was determined by the biuret method as modified for protein determination of whole cells by HERBERT and coworkers (1971a).

1.6. Carbohydrate determination

Reducing sugars were measured by the method of SOMOGYI (1952), while total carbohydrate contents of the fermentation broths were determined by the phenol method (HERBERT *et al.*, 1971b).

1.7. Kinetic analysis

Growth and protein formation of the culture was kinetically analysed in the case of regular growth curves according to KONO (1968) and KONO and ASAI (1968). The kinetic analysis of diauxic growth was performed using the method of KONO and ASAI (1971), while product formation of the diauxic cycle was calculated according to our previous publication (ZETELAKI-HORVÁTH, 1972).

2. Results

The mycelial and protein yields of the *Mucor mucedo* strain (No. 224) as well as the rate of growth and of protein formation, in synthetic glucose media (**No. 1**), were plotted in Fig. 1 and Table 1.

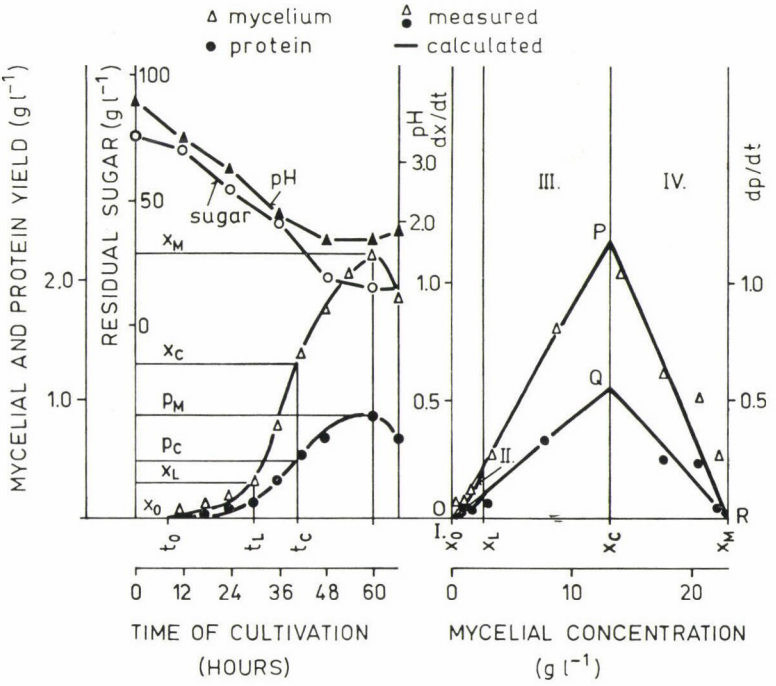

Fig. 1. Mycelial and protein yields and rates of growth and protein formation of the *Mucor mucedo* culture (No. 224) grown in synthetic glucose (**No. 1**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-1})

Table 1

Kinetic constants of the Mucor mucedo strain (No. 224)

Media	Part of diauxie	t_0	t_L	t_C	$t_{M/x}$	x_0
No. 1	1	9	30	40.5	60	0.0
Glucose						
No. 2	1	0	11	17.5	30	0.0
Glucose + corn-steep	2	27	31.5	40.0	50	9.5
No. 3	1	6	9	15	27.5	0.0
Sucrose	2	24	30	40	54	7.0
No. 4	1	6	12	16	25.5	0.0
Sucrose + corn-steep	2	24	27.5	39	66	8.0
No. 5						
Bread	1	3	10	20	60	0.0

The pH and the sugar content of the media decreased till the 60th hour of fermentation, then an increasing tendency was observed in the pH.

The growth curve of the culture was a simple oneconsisting of fourphases. A rather long transient phase is the characteristic of the curve. While the lag phase lasted for 9 hours, the transient phase continued more than twice as long (21 hours).

The rates of growth and protein formation in the exponential phase proved to be 0.092 and 0.042 h^{-1}, resp. Maximum mycelial and protein yields were attained in cultures of the same age (22.2 and 9.6 g l^{-1}, resp.).

The mycelial and protein yields of the *Mucor mucedo* culture grown in semi-synthetic glucose medium (**No. 2**) were plotted as a function of time of cultivation while data of rates of growth and of protein formation were plotted against concentration of mycelia, in Fig. 2. Data of the kinetic constants and the rates of growth and product formation are given in Table 1.

In this semi-synthetic medium diauxic growth of the culture was found. Both the first and second stage of the diauxie lasted for 27 hours. At the time of the first declining phase of the diauxie, breaking points were observable in the decreasing tendency of pH and residual sugar, resp. Protein formation of the culture proved to be growth-related. Growth and protein formation were more intensive in the second part of the diauxic cycle. The rates of growth in the first and second exponential phases were 0.150 and 0.066 h^{-1}, while the rates of protein formation: 0.083 and 0.026 h^{-1}, resp.

Maximum mycelial and protein yields were obtained in the 54-hour culture (26.3 and 10.8 g l^{-1} resp.).

grown in synthetic, semi-synthetic and natural media

x_L	x_C	x_M	k	k_{p_1}	k_{p_2}	p_C	p_M	$t_{M/p}$
3.0	13.0	22.2	0.092	0.042	0.00	4.8	9.6	60
1.0	6.0	10.0	0.150	0.083	0.00	3.0	0.0	54
12.0	21.0	26.3	0.066	0.026	0.00	9.0	10.8	
1.0	5.0	8.4	0.170	0.060	0.00	2.5	0.0	54
9.4	17.0	24.0	0.085	0.032	0.00	7.4	9.0	
1.0	5.0	8.6	0.200	0.100	0.00	2.0	0.0	54
10.0	19.0	27.0	0.063	0.029	0.00	8.6	11.0	
3.0	13.0	34.0	0.106	0.060	0.00	6.3	16.0	54

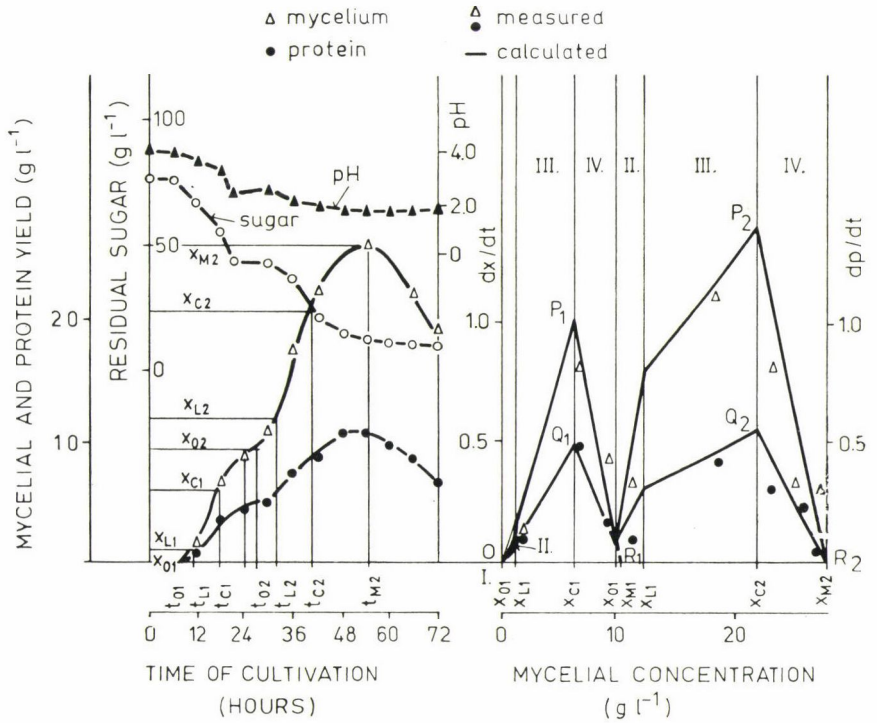

Fig. 2. Mycelial and protein yields and rates of growth and protein formation of the *Mucor mucedo* culture (No. 224) grown in semi-synthetic glucose (**No. 2**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 $l\,l^{-1}\,min^{-1}$; O_2-solution rate: 50.8 mmole $l^{-1}\,h^{-1}$)

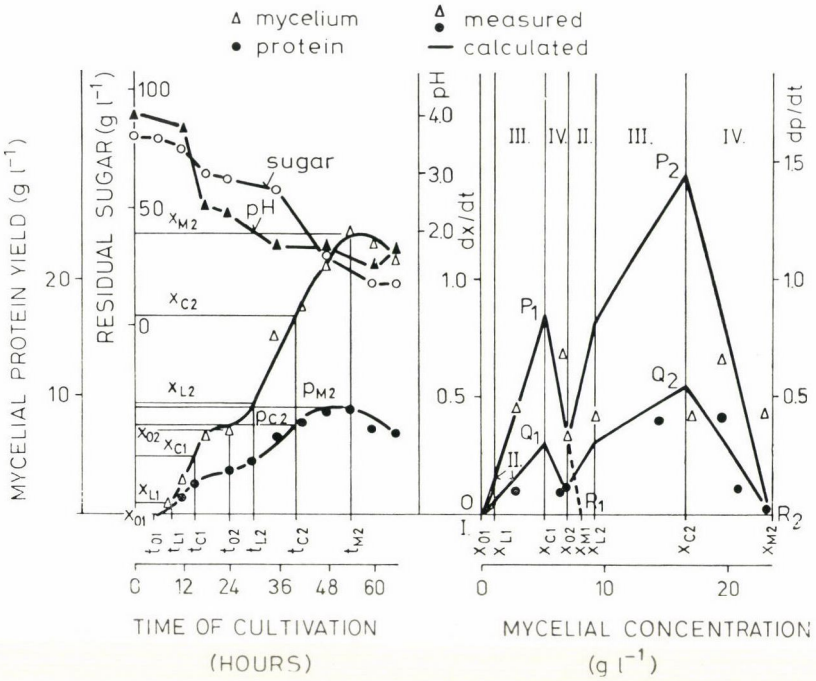

Fig. 3. Mycelial and protein yields and rates of growth and protein formation of the *Mucor mucedo* culture (No. 224) grown in synthetic sucrose (**No. 3**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-1})

The mycelial and protein yields as well as the rates of growth and protein formation of the above *Mucor mucedo* strain in synthetic sucrose medium (**No. 3**) can be seen in Fig. 3. Kinetic constants of the culture can also be found in Table 1.

The growth of the above strain proved to be diauxic in sucrose-containing synthetic medium. The pH and the sugar content of the fermentation broth decreased till the 54th hour of the fermentation, except for the break which occurred in the period of the first declining phase.

The first part of the diauxic cycle lasted for 24 hours while the second one for 30 hours. The rates of growth in the first and second exponential phases were 0.170 and 0.085 h^{-1} and the rates of protein formation: 0.060 and 0.032 h^{-1}, resp.

The protein formation of the culture proved to be growth-related in both parts of the diauxic cycle.

Maximum mycelial and protein yields were obtained in the 54-hour culture which were 24 and 9 g l^{-1}, resp.

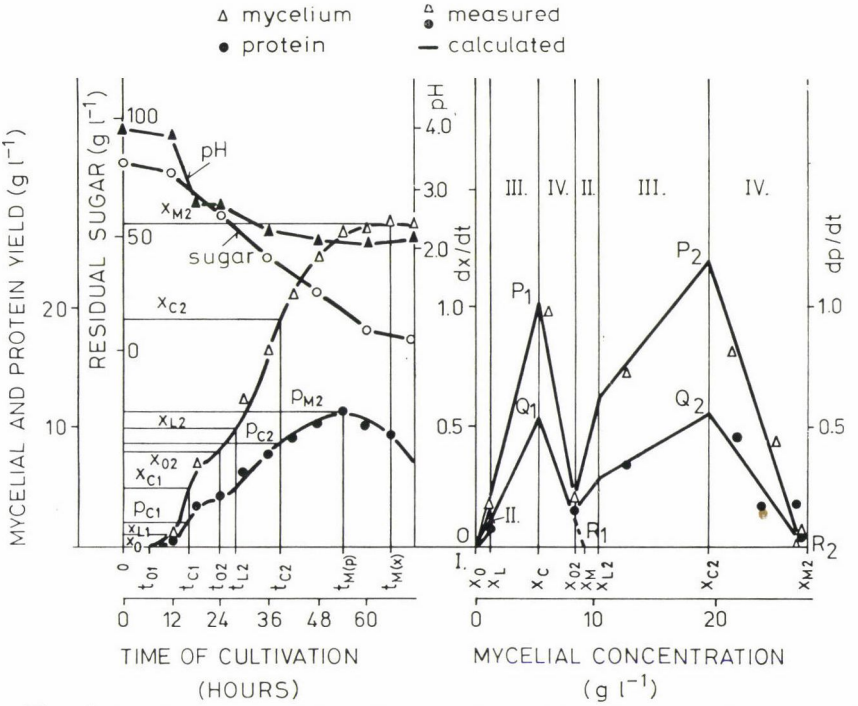

Fig. 4. Mycelial and protein yields and rates of growth and protein formation of the *Mucor mucedo* culture (No. 224) grown in semi-synthetic sucrose (**No. 4**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-1}).

Growth and protein formation of the strain were also tested in sucrose medium supplemented with corn-steep liquor (medium **No. 4**). Kinetic data and the growth of the culture can be found in Table 1 and Fig. 4.

The growth of the culture was also diauxic when cultivated in the above semi-synthetic sucrose medium.

The pH values of the fermentation broth decreased in the exponential phases and slightly increased in the declining phases. The sugar content of the medium decreased continuously till the end of the fermentation.

It is a characteristic of the growth curve that the first part of the diauxie is short (23 hours), while the second one long (48 hours). The mycelia synthesized in the second part are more than twice as much as those synthesized in the first stage.

The protein formation was growth-related in both parts of the diauxic cycle.

The rates of growth in the first and second exponential phases were 0.200 and 0.063 h^{-1}, and the rates of protein formation: 0.100 and 0.029 h^{-1}, resp. Maximal mycelial yield (27 g l^{-1}) was attained in the 66-hour culture while maximal protein yield (11.03 g l^{-1}) in the 54-hour culture.

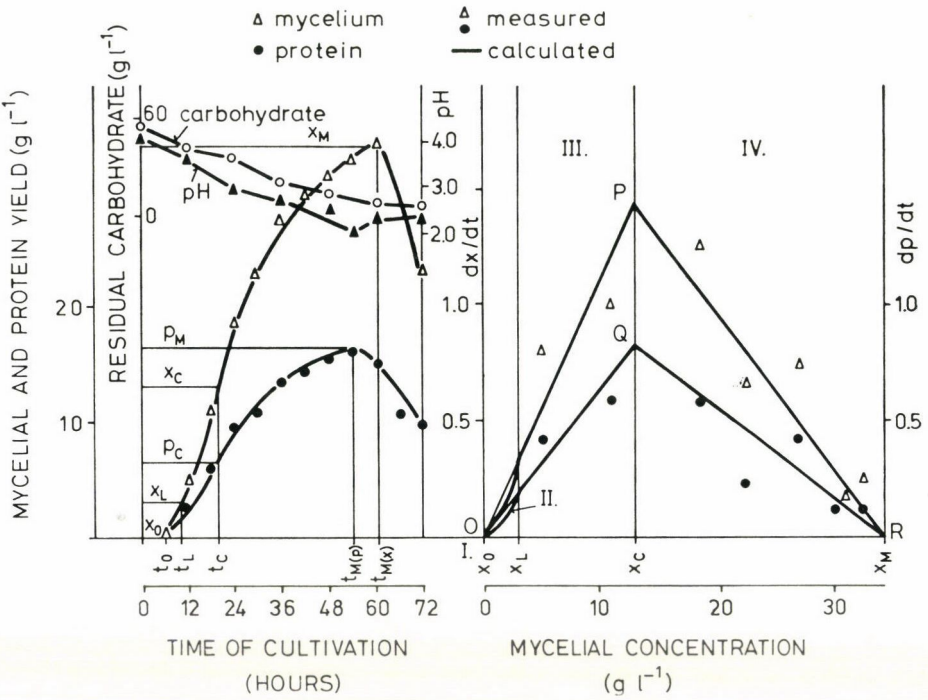

Fig. 5. Mycelial and protein yields and rates of growth and protein formation of the *Mucor mucedo* culture (No. 224) grown in natural bread (**No. 5**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-1})

As the yields of the *Mucor mucedo* culture could be increased by changing over from the synthetic media to semi-synthetic ones, the effect of natural media (bread medium **No. 5**) on the cell and protein synthesis of the culture was also tested. Though ground bread can easily be utilized by the mould, hydrolysed bread was used, in order to avoid the errors in measuring mycelial yield in young cultures due to unsolubilized bread particles. The mycelial and protein yields and the rates of growth and protein formation of the culture grown in natural media are given in Fig. 5 and Table 1.

As can be seen in the figure, cultivation of the above strain in natural medium resulted in a regular growth. The growth curve consisted of four phases. Its characteristic was a very long (40-hour) declining phase.

pH values of the culture showed the same tendency (decreasing in the exponential and increasing in the declining phase) as was observed with other media.

The rates of growth and protein formation in the exponential phase were 0.106 and 0.060 h^{-1}, resp. The protein formation of the culture was growth-related.

Maximum mycelial yield (34 g l^{-1}) was obtained in the 60-hour culture, while the highest protein yield (16 g l^{-1}) in the 54-hour culture.

3. Conclusions

Comparing the type of growth of the culture in the above media, a regular growth curve of four phases was found when synthetic glucose (**No. 1**) and natural bread (**No. 5**) resp., were used as the carbon source. In agreement with our previous results (ZETELAKI-HORVÁTH *et al.*, 1973) diauxic growth occurred in the cultures grown in semi-synthetic and in synthetic sucrose media. If diauxie occurred, the greater part of the mycelium yield was synthesized in the second cycle. The rates of growth in the first exponential phase were found to be two-fold or more than those in the second one. The highest rate of growth (0.200 h^{-1}) was found in a culture grown in semi-synthetic sucrose medium (**No. 4**) followed, in decreasing sequence, by the synthetic sucrose (**No. 3**, 0.170 h^{-1}) and the semi-synthetic glucose (**No. 2**, 0.150 h^{-1}) medium.

The *Mucor mucedo* strain (No. 224) grew according to a simple curve consisting of four phases when incubated in synthetic glucose medium (**No. 1**). The characteristic of the curve was a rather long (21-hour) transient phase. The rate of growth (k) in the exponential phase proved to be 0.092 h^{-1}.

A four-phase, simple growth curve was also found when the culture was grown in natural bread medium. The characteristic of the growth curve of the culture in bread medium is a long (40-hour) declining phase. The rate of growth of the culture in the exponential phase was found to be 0.106 h^{-1}.

In contrast to our previous work (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973) when different types of enzyme-protein synthesis were found, the formation of cell protein proved to be growth-related in the cases of each media used.

The rates of protein formation (k_{p1}) were higher in the cultures grown according to diauxie than in the ones having simple four-phase growth. In the case of diauxie the highest rate of protein formation occurred in the first exponential phases of the diauxic cycles. The highest rate of protein formation (0.100 h^{-1}) was found in the semi-synthetic sucrose medium (**No. 4**) following that, in decreasing sequence, the semi-synthetic glucose (**No. 2**) and the synthetic sucrose (**No. 3**) media (0.083 and 0.060 h^{-1}, resp.). The rate of protein formation of the culture grown in bread medium was 0.060 h^{-1}.

The highest mycelial yield (34 g l^{-1}) was attained in the 60-hour *Mucor*-culture grown in natural bread medium. The highest protein yield was also found when the culture was grown in the same medium but in a shorter (54-hour) time of incubation.

The highest mycelial yield of the cultures grown in diauxic cycle proved to be 27 g l^{-1} after 66 hours of fermentation and the highest protein yield of 11.03 g l^{-1} under 54 hours of fermentation. The protein yields of the cultures incubated in synthetic glucose and sucrose media were found to be less than 10 g l^{-1}.

*

The authors thank Mrs. E. NYERGES-ROGRÜN of the NATIONAL RESEARCH INSTITUTE FOR VITICULTURE AND ENOLOGY, Budapest, for the mould culture.

NOMENCLATURE

k	growth rate constant, h^{-1}
k_{p1}	production rate constant of the growing cells, h^{-1}
k_{p2}	production rate constant of the resting cells, h^{-1}
t	time, h
x	cell concentration, g l^{-1}
p	protein concentration, g l^{-1}

Subscripts

O	refers to the boundary of an induction phase and a transient phase
L	refers to the boundary of a transient phase and an exponential growth phase
C	refers to the critical point, that is the boundary of an exponential growth phase and a declining growth phase
M	refers to theoretical maximum value of cell and product concentration, resp. The slopes of $\overline{OP}$, $\overline{OQ}$ and $\overline{OR}$ represent the values of k, k_{p1}, and k_{p2}, resp.
$t_{M/x}$	time of maximum mycelium yield
$t_{M/p}$	time of maximum protein yield

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KINETIC ANALYSIS OF PROTEIN SYNTHESIS IN FUNGI

PART III. — THE EFFECT OF COMPOSITION OF MEDIA ON GROWTH AND PROTEIN FORMATION OF *RHIZOPUS COHNII*

K. ZETELAKI-HORVÁTH and K. VAS

(Received September 4, 1975)

Mycelial and protein synthesis of a *Rhizopus cohnii* strain (No. 222) was studied under submerged conditions in synthetic, semi-synthetic and in natural media.

Growth and protein formation of the above strain were kinetically analysed. The culture grew according to a regular growth curve, consisting of four phases, when cultivated in glucose and bread media (media **Nos. 1** and **5**, resp.) and diauxic growth was obtained when cultivated in glucose and corn-steep, sucrose, and sucrose plus corn-steep media, resp. (**Nos. 2**, **3** and **4**).

The protein formation of the culture proved to be growth-associated in each of the media used.

The highest rate of growth (0.160 h^{-1}) was found in the first exponential phase of the cultures grown in media **Nos. 2** and **3** and the highest rate of protein formation (0.080 h^{-1}) in medium **No. 3.**

The highest protein yields (13.0 and 11.7 g l^{-1}) were obtained in bread (**No. 5**) and in semi-synthetic sucrose medium (**No. 4**) after 42 and 72 hours of cultivation, resp. Maximal protein yield (10.4 g l^{-1}) was measured in the 60-hour culture when incubated in synthetic glucose medium (**No. 1**), while in semi-synthetic glucose (**No. 2**) and in synthetic sucrose (**No. 3**) media (7.2 and 9.8 g l^{-1}, resp.) the maximum protein yield was found after 48 hours of fermentation.

1. Materials and methods

1.1. Microorganism

A *Rhizopus cohnii* strain (No. 222) was used as a test organism (from the strain collection of the NATIONAL RESEARCH INSTITUTE FOR VITICULTURE AND ENOLOGY). Stock cultures were maintained on agar slants.

1.2. Nutrient media

For *inoculation* the following composition of media was used: yeast 5.0 g (in the form of an extract); soluble starch 15 g; KH_2PO_4 1.0 g and $MgSO_4 \cdot 7H_2O$ 0.5 g adjusted to 1 000 ml by tap water, pH to 5.5. As *fermentation* media synthetic (**Nos. 1** and **3**), semi-synthetic (**Nos. 2** and **4**) and natural media (**No. 5**) were used: **No. 1**: glucose 80 g; **No. 2**: glucose 80 g; corn-steep liquor 20 g; **No. 3**: sucrose 80 g; **No. 4**: sucrose 80 g; corn-steep liquor 20 g; **No. 5**: ground bread 40 g. The method of bread hydrolysis was given elsewhere (ZETELAKI-HORVÁTH *et al.*, 1975). After hydrolysis the pH was read-

justed to 4.0 with NaOH. Minerals were given to each of the above media as follows: $(NH_4)_2SO_4$ 10 g; $ZnSO_4$ 0.25 g; $MnSO_4$ 0.10 g; KH_2PO_4 1.0 g, the volume was adjusted to 1 000 ml with tap water, pH to 4.0.

1.3. Method of cultivation

100 ml of inoculum media were inoculated with 1 ml of spore suspension and was incubated for 24 hours on a rotary shaker (rpm 330; stroke 20 mm; O_2-transfer rate in the flasks 17–19 mmole O_2 l^{-1} h^{-1}) at a temperature of 28°C. The fermentations were carried out in 10-liter glass fermenters. The 6 000 ml medium contained in a fermenter was inoculated with 600 ml of the above 24-hour vegetative culture. The speed of agitation was 460 rpm, and the volume of air that was bubbled through the cultures was 1.0 l l^{-1} min^{-1}. The oxygen transfer rate in the fermenter was 50.8 mmole O_2 l^{-1} h^{-1} determined by the method of COOPER and co-workers (1944).

1.4. Preparation of the mycelia

Mycelia were obtained from the fermentation broth by filtration through a Nylon cloth. The residues of the medium were removed by thorough washing in water, then the mycelia were dried at 105°C to constant weight.

1.5. Protein determination

The protein content of the mycelia was determined by the biuret method, modified for the protein determination of whole cells by HERBERT and co-workers (1971a).

1.6. Carbohydrate determination

Reducing sugars were measured by the method of SOMOGYI (1952) while total carbohydrate contents of the fermentation broths were determined by the phenol method (HERBERT and co-workers, 1971b).

1.7. Kinetic analysis

In the case of regular growth curves kinetic analysis of growth and protein formation were made according to the equations of KONO (1968) and KONO and ASAI (1968). Kinetic analysis of diauxic growth was made by the method of KONO and ASAI (1971), and the product formation of diauxic cycle according to our previous work (ZETELAKI-HORVÁTH, 1972).

2. Results

The mycelial and protein yield together with the rate of growth and of protein formation of the above strain when cultivated in synthetic glucose medium (**No. 1**) are given in Fig. 1 and Table 1.

The growth curve of the strain can be divided into four phases. The lag (I) and the transient phase (II) of the culture were too long (15 and 28.5 hours, resp.). The growth started at a rather slow rate. Maximal mycelium (18.2 g l^{-1}) and protein yields (10.4 g l^{-1}) were obtained in the 60-hour culture. The pH-value of the media decreased to the time of the maximum mycelial yield, showing an increasing tendency later. The initial glucose concentration (80 g l^{-1}) decreased to 22 g l^{-1} until the end of the cultivation. The rates of growth and of protein formation were 0.135 and 0.064 h^{-1}, resp.

The mycelial and protein yields as well as the rates of growth and protein formation of the above culture grown in medium containing glucose and corn-steep liquor (medium **No. 2**) are shown in Fig. 2 and Table 1.

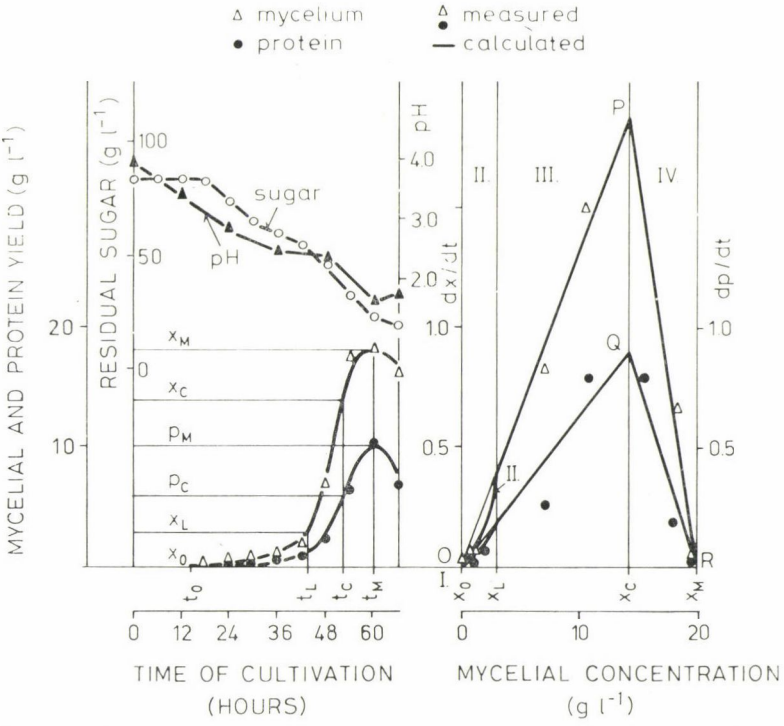

Fig. 1. The mycelial and protein yield and the rates of growth and protein formation of the *Rhizopus cohnii* culture grown in synthetic glucose (**No. 1**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-1})

Table 1

Kinetic constants of the Rhizopus cohnii strain

Media	Part of diauxie	t_O	t_L	t_C	$t_{M/x}$	x_O
No. 1						
Glucose	1	15	43.5	52.5	60	0.0
No. 2	1	6	9	16	34	0.0
Glucose + corn-steep	2	31	37	45	48	8.4
No. 3	1	6	9	16	33	0.0
Sucrose	2	30	36	41	48	8.0
No. 4	1	6	10.5	21	37.5	0.0
Sucrose + corn-steep	2	39	45	58	66	9.6
No. 5						
Bread + corn-steep	1	6	10.5	18.5	54	0.0

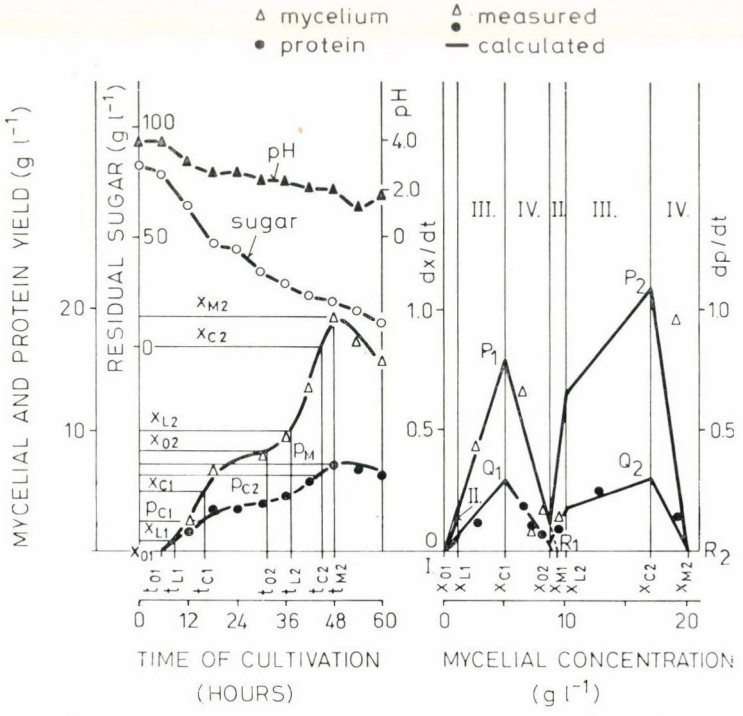

Fig. 2. The mycelial and protein yield and the rates of growth and protein formation of the *Rhizopus cohnii* culture grown in semi-synthetic glucose (**No. 2**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-1})

grown in synthetic, semi-synthetic and in natural media

x_L	x_C	x_M	k	k_{p_1}	k_{p_2}	p_C	p_M	$t_{M/p}$
3.0	14.0	18.2	0.135	0.064	0.00	6.0	10.4	60
1.0	5.0	9.0	0.160	0.060	0.00	2.5	0.0	0
10.0	17.0	19.2	0.064	0.017	0.00	6.4	7.2	48
1.0	5.0	8.5	0.160	0.080	0.00	3.0	0.0	0
10.0	17.0	20.0	0.088	0.038	0.00	8.0	9.8	48
1.0	5.6	9.2	0.125	0.056	0.00	2.8	0.0	0
12.0	23.0	28.4	0.054	0.024	0.00	8.8	11.7	66
2.5	13.0	29.5	0.126	0.060	0.00	6.5	13.0	42

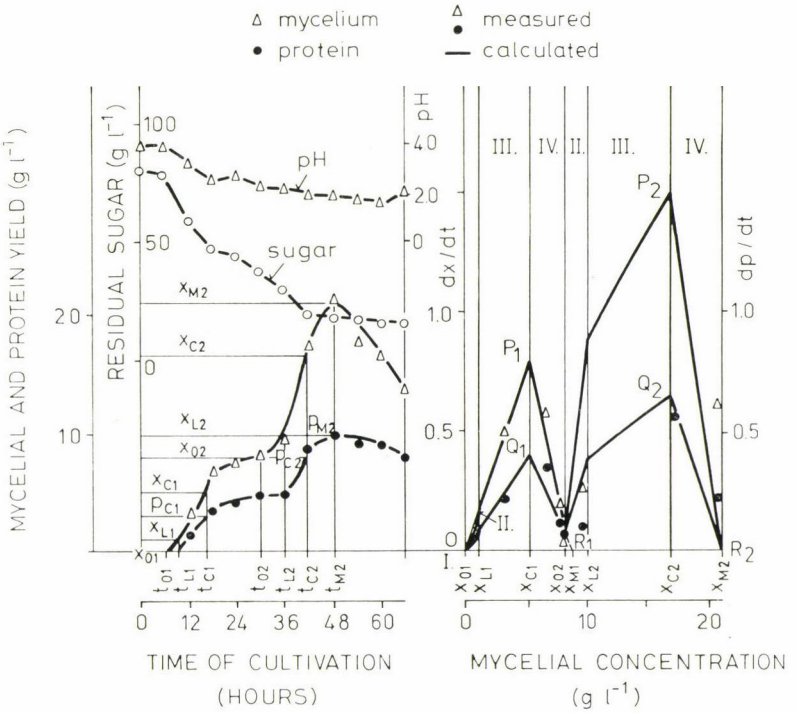

Fig. 3. The mycelial and protein yield and the rates of growth and protein formation of the *Rhizopus cohnii* culture grown in synthetic sucrose (**No. 3**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-11})

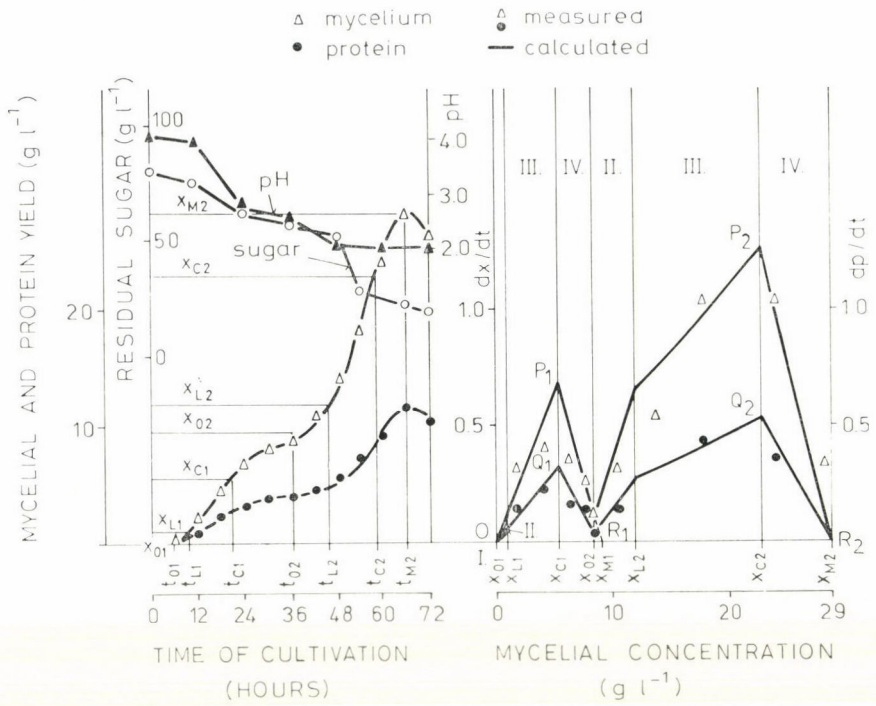

Fig. 4. The mycelial and protein yield and the rates of growth and protein formation of the *Rhizopus cohnii* culture grown in semi-synthetic sucrose (**No. 4**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-1})

According to Fig. 2 the type of growth of the culture proved to be diauxic in a medium containing corn-steep liquor. Maximum mycelial and protein yields of the strain were attained in the 48-hour culture (19.2 and 7.2 g l^{-1}, resp.). The declining phase of the growth in the first part of the diauxie was accompanied by a decreased sugar consumption and a slight increase in the pH value.

The rates of growth of the culture in the first and in the second part of the diauxie were 0.160 and 0.064 h^{-1}, while the rates of protein formation proved to be 0.060 and 0.017 h^{-1}, resp.

The mycelial and protein yields of the *Rhizopus cohnii* (No. 222) culture together with the rate of growth and of protein formation in synthetic sucrose medium (medium **No. 3**) can be seen in Fig. 3.

According to the data of Fig. 3, the growth of the above strain in synthetic sucrose media was found to be diauxic, too. A rather long declining phase was developed in the first part of the diauxie with a decreased sugar consumption and increasing pH-values. The growth in the second exponential phase was intensive. Maximum mycelial and protein yields were obtained in the 48-hour culture (20.0 and 9.8 g l^{-1}). The rates of growth in the first and second

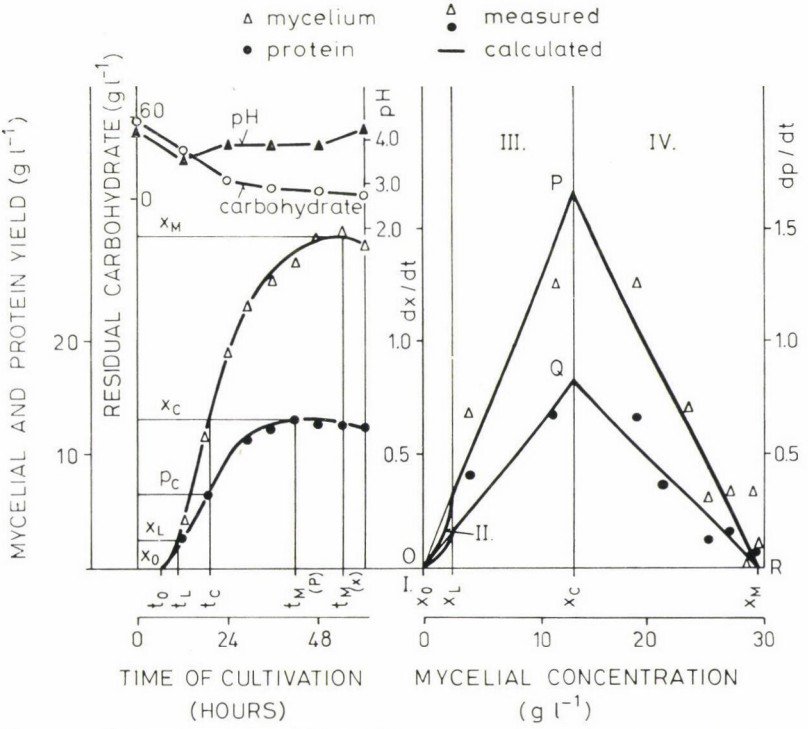

Fig. 5. The mycelial and protein yield and the rates of growth and protein formation of the *Rhizopus cohnii* culture grown in natural bread (**No. 5**) medium. (Agitation speed: 460 rpm; aeration rate: 1.0 l l^{-1} min^{-1}; O_2-solution rate: 50.8 mmole l^{-1} h^{-})

exponential phases were found to be 0.160 and 0.088 h^{-1}, while the rates of protein formation were 0.080 and 0.038 h^{-1}, resp.

The growth and protein formation of *Rhizopus cohnii* in medium containing sucrose and corn-steep liquor (medium **No. 4**) was also tested. The mycelial and protein yields were plotted against time of cultivation, while rates of growth and of protein formation as functions of mycelial concentration in Fig. 4. Data of rates of growth and protein formation, together with the kinetic constants of the culture, are given in Table 1.

The growth of the above strain was diauxic again. The first part of the diauxic cycle lasted for 39 hours, while the second part for 27 hours. Mycelial formation of the culture was very intensive in the second cycle and more than twice as much mycelia (19.2 g l^{-1}) were synthesized than in the first cycle (9.2 g l^{-1}). The rates of growth in the first and second exponential phases were 0.125 and 0.054 h^{-1}, resp., while the rates of protein formation were 0.056 and 0.024 h^{-1}, resp.

Maximum mycelial and protein yields were attained in the 66-hour culture (28.4 and 11.7 g l^{-1}, resp.).

The effect of natural media (containing bread; medium **No. 5**) on the growth and protein formation of *Rhizopus cohnii* was also investigated. Mycelial and protein yields were plotted as functions of time of cultivation while rates of growth and protein formation were given as functions of mycelial concentration in Fig. 5. Data of rates of growth and protein formation with the kinetic constants of the culture are listed in Table 1.

The pH of the fermentation broth showed a very slight change during cultivation, a decrease in the first 12 hours. In the second 24 hours the pH remained constant, increasing thereafter till the end of the fermentation.

Figure 5 shows that the *Rhizopus*-culture grew according to a simple growth curve consisting of four phases in bread media.

The rates of growth and protein formation were 0.126 and 0.060 h^{-1}, resp.

Maximum mycelial yield (29.5 g l^{-1}) was attained in the 54-hour culture, while maximal protein yield (13.0 g l^{-1}) in the 42-hour culture.

3. Conclusions

The cultivation of the *Rhizopus cohnii* strain (No. 222) in media of various carbon sources with and without corn-steep liquor resulted in different types of growth. In agreement with our previous observation (ZETELAKI-HORVÁTH *et al.*, 1973) diauxic growth occurred when cultures were grown in media containing glucose and corn-steep liquor, and sucrose with and without corn-steep liquor.

When a glucose medium was used without corn-steep liquor and a natural medium containing bread, cultures were grown according to a one-stage growth curve consisting of four phases.

The lag phase of the cultures grown in the above media lasted 6 hours, with the exception of glucose medium, where a 15-hour long lag-phase was found. The rates of growth in the exponential growth phases of the culture were the highest (0.160 h^{-1}; in media containing glucose and corn-steep liquor and sucrose without corn-steep liquor) in the first stage of the diauxie. With the use of media **Nos. 1** and **5**, cultures showed a simple growth curve of four phases and the rates of growth were found to be 0.135 and 0.126 h^{-1}, resp. (Table 1).

Mycelial yields of the culture attained in bread and sucrose medium containing corn-steep liquor were higher (29.5 and 28.4 g l^{-1}, resp.) than those in synthetic glucose, sucrose and in semi-synthetic glucose media (18.2, 20.0 and 19.2 g l^{-1}, resp.). The highest mycelial yield of the culture was attained under the shortest cultivation time (54 hours) in bread medium.

In spite of our previous results related to the type of enzyme protein

synthesis (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1973) protein formation proved to be growth-associated and this did not vary with the change of the composition of the media. The curves representing the protein yields were similar to those of the growth curve. In cultures where the cell synthesis was diauxic protein synthesis was diauxic, too.

The highest rate of protein formation (0.080 h^{-1}) was found in the first stage of the diauxic cycle when the *Rhizopus*-culture was grown in media containing sucrose.

Final purpose of this work was the production of protein by *Rhizopus*-culture, and results must be evaluated from the viewpoint of the protein yield as well.

Highest protein yield (13.0 g l^{-1}) was obtained in the shortest time (42 hours) in the culture grown in natural (bread) medium. In media containing sucrose and corn-steep liquor 11.7 g l^{-1} protein yield was attained but only 30 hours later.

A rather short time was necessary for the production of maximal protein yields in media **Nos. 2** and **3** (glucose and corn-steep and sucrose without corn-steep) but these yields were much lower (7.2 and 9.8 g l^{-1}) than the previous ones.

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The authors are indebted to Mrs. E. NYERGES-ROGRÜN, NATIONAL RESEARCH INSTITUTE FOR VITICULTURE AND ENOLOGY, Budapest, for placing the mould culture at their disposal.

NOMENCLATURE

k	growth rate constant, h^{-1}
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k_{p2}	production rate constant of the resting cells, h^{-1}
t	time, h
x	cell concentration, g l^{-1}
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Subscripts

O	refers to the boundary of an induction phase and a transient phase
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M	refers to theoretical maximum value of cell and product concentration, resp. The slopes of $\overline{OP}$, $\overline{OQ}$ an $\overline{OR}$ represent the values of k, k_{p1} and k_{p2}, resp.
$t_{M/x}$	time of maximum cell concentration
$t_{M/p}$	time of maximum product concentration

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RESISTANCE OF *BACILLUS CEREUS* SPORES AS AFFECTED BY CHANGES IN THEIR EXCHANGEABLE Ca^{2+} CONTENT*

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Based on knowledge of the ionic state of spores, the effect of treatments presumably inducing cation exchange in the spores of two *Bacillus cereus* strains was investigated. An assay was made to convert the spores grown on tryptone–yeast broth–glucose agar into their H^+ form by incubation in hydrochloric acid of pH 3.5 or to obtain Ca^{2+} form spores by incubation in an 0.02 *M* Ca-acetate solution adjusted to pH 8.0. The effect of the chemical treatments on the viability, germinating capacity and thermoresistance of the spores was investigated. Further, changes in the dipicolinic acid (DPA) content of spores as induced by ion-exchange incubation and subsequent heat treatments were studied. The same experiments were carried out with spores propagated on media of high Ca content (0.02 *M*). The thermoresistance of the majority of spores exposed to acid treatment corresponded practically to the original resistance of the spores, when not exposed to ion-exchange incubation. In the acid-treated spores, however, the super-resistant fraction, causing the tailing of the survival curve, was rarely found. The thermoresistance in the fraction of Ca-acetate-treated spores, which did not germinate or survived the treatment, substantially increased. Calcium treatment caused in the majority of the spores a change similar to germination and this seems to support the hypothesis known as the "expanded spore cortex" theory. The kinetics of thermal death appeared practically the same in spores converted to their Ca^{2+} form as in spores grown on media rich in Ca. The difference in the thermoresistance of the H^+ and Ca^{2+} form spores remained unchanged during the 6-month refrigerated storage of their suspension in water. The loss in dipicolinic acid was found to be proportional to the germination and thermal death of the spores. The kinetics of dipicolinic acid exudation showed a nearly linear character as a function of time and not an exponential one.

The thermoresistance of bacterial spores, highly exceeding that of vegetative cells, is an extremely important problem of food microbiology. The termination of the dormant state, the transformation of spores into vegetative cells, *i. e.* their germination, entails, already in its early stage, the loss in thermoresistance. Therefore the knowledge of determinants responsible for dormancy and thermoresistance is a crucial point in a more efficient defence against spore contamination.

Studies into this problem have already shown the spores to be of a more intricate structure than the vegetative cells. Out of the layers enveloping the spore-protoplast, the one called cortex, consisting of a loose network of peptidoglycan polymer, is considered to play an important role in the retention of the

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spore state and of thermoresistance. A further characteristic of spores is their abundance in divalent metal ions. Calcium is dominant among the metal ions, reaching frequently a concentration amounting to 2% of spore solids. Dipicolinic acid (2,6-pyridine dicarboxylic acid) of chelating capacity forms about 5 to 15% of spore solids, while not present at all in vegetative cells. It has been shown that these spore components or the conditions leading to their accumulation are of major importance in the heat resistance of spores (POWELL, 1953; CHURCH & HALVORSON, 1959; LECHOWICH & ORDAL, 1960; LEVINSON *et al.*, 1961; RIEMANN, 1961; CHUNG *et al.*, 1971).

In the course of this study the effect of the Ca content of the medium used in surface propagation of two different *Bacillus cereus* strains on their thermoresistance was investigated. Beside the death rate as caused by heat treatment the loss of dipicolinic acid was followed up.

During the last decade ALDERTON and co-workers (1964) have shown, as a result of their experiments with the spores of *B. megaterium* and *B. stearothermophilus*, that thermoresistance cannot be considered an inherent, unchangeable property of bacterial spores, because it is liable to reversible changes. The spores behaved as cation exchangers; in an acid medium a part of their Ca^{2+} content was substituted for H^+ ions and as a consequence their thermoresistance was substantially reduced. In mildly alkaline Ca-acetate buffer, however, the H^+-form spores could be re-formed into Ca^{2+}-form spores (ALDERTON *et al.*, 1964; ALDERTON & SNELL, 1963; 1969a, b; 1970).

It was investigated, therefore, whether the same behaviour could be proven in *Bacillus cereus* spores. The thermoresistance of the spores and changes in their dipicolinic acid (DPA) and Ca content during ion exchange and subsequent heat treatments were also studied.

1. Materials and methods

1.1. Microorganisms

Bacillus cereus T originating from UNILEVER RESEARCH LABORATORY (Sharnbrook, Bedford, U.K.) and a *Bacillus cereus* P. 715 strain isolated at the CENTRAL FOOD RESEARCH INSTITUTE, Budapest, from peas were used as test organisms.

1.2. Preparation of the spore suspensions

The spores were produced in Petri-dishes on a nutrient agar containing 0.5% tryptone, 0.25% yeast extract 0.15% glucose, 1.5% agar-agar, adjusted to pH 7.0 and sterilized at 121 °C for 15 min. A 24-hour culture of vegetativ

cells, obtained on a nutrient medium containing 2% trypton, 0,25% glucose, 0.5% NaCl, 0.25% K_2HPO_4 (pH 7.3) was used as the inoculum. The vegetative cells were streaked on the surface of the nutrient agar and subsequently incubated for 72 hours at 30°C. The spores thus formed were kept with the nutrient agar for 18 hours at 4°C. Then the spores were aseptically collected and suspended in sterile distilled water. According to FRIEDMAN and GRECZ (1973) 0.1 mg ml^{-1} lysozyme was added to the suspension and it was stored at 4°C overnight. The spore mass was washed ten times by suspending in cooled sterile distilled water and centrifuging at 4°C in a *Beckman* J-21 apparatus of 7 000 rpm for 15 min. Finally, the 50 Petri-dishes of 9 cm diameter were used to prepare 50 ml spore suspension and this was stored in a refrigerator till used. The medium rich in Ca was of the same composition, only 20 m*M* $CaCl_2$ were added to it prior to sterilization. Thus the Ca concentration of the medium was increased sevenfold, from 3.7 m*M* to 24.5 m*M*. The pH was adjusted to 7.0 and the procedure continued as above.

1.3. Thermal death

Thermal death was determined at 90°C in a water bath of ± 0.005°C accuracy (*Braun Thermomix* 1460). The spore suspension in distilled water, used in the experiments, had a density of 10^8 spores per ml. Each test tube contained 10 ml suspension and the heat treatment period was extended by the predetermined heating-up period. The viable cell count was determined by the MPN technique in a nutrient containing 0.1%, meat broth 0.5%, pepton 0.2%, yeast extract 0.5%, NaCl at pH 7.4 and sterilized at 121°C for 15 min, in five replicates each. The test tubes were incubated at 30°C.

1.4. Determination of dipicolinic acid

The spore exudates were investigated by mixing 5 ml spore suspension with 0.1 ml *N* acetic acid and centrifuging the mixture after an hour in a *Beckman* J-21 apparatus at 10 000 rpm for 15 min. The DPA in the supernatant was determined according to JANSSEN and co-workers (1958): 1 ml freshly prepared reagent was added to 3 ml clear solution. The composition of the reagent is as follows: 0.1% cysteine, 1.0% $Fe(NH_4)_2(SO_4)_2\ 6H_2O$ (*Mohr* salt), dissolved in 0.05 *M* acetate buffer of pH 4.0. The yellow colour thus developed was measured within 2 hours in a *Gilford* 2400 spectrophotometer at 440 nm. The DPA concentration was established by comparison to a calibration curve plotted with analytical grade DPA. The total DPA content of spores was understood to be the amount of DPA obtained by heating the aqueous suspension of the spores to 1 atm gauge pressure for 15 min.

1.5. Determination of the Ca content

The Ca content was determined from the same supernatant, given acetic acid treatment, by complexometric titration (SCHWARZENBACH & FLASCHKA, 1968). The titration was carried out in a *Radiometer* phototitrimeter with a 0.03 *M* Na_2EDTA solution in the presence of murexide indicator. The phototitrimeter equipment consisted of the following accessories: *Titrigraph* SBR 2c, *Titrator* TTT 11, *pH Meter* 26, *Autoburette* ABU 12, *Titration Assembly* TTA4.

1.6. Ion exhange chemical treatment of the spores

The spores obtained on the basal medium of tryptone–yeast extract–glucose were incubated in a HCl solution of pH 3.5 at 60°C for 1 hour, followed by treatment for 3 hours at 25°C to convert them into their H^+ form. To obtain Ca^{2+} form spores they were incubated overnight at 60°C in a 0.02 *M* Ca-acetate solution, adjusted to pH 8.0. After acidification the pH was re-adjusted to pH 6.5 with NaOH solution. Both spore suspensions given different chemical treatments, were then three times centrifuged and washed with distilled water.

2. Results

2.1. Thermoresistance of the spores as affected by enrichment of the spore-forming nutrient in Ca

Both strains were applied in four parallel thermal death experiments. Fig. 1 shows the average values of results obtained by the MPN method and the standard errors as a function of the thermal treatment period. Beside the survival curves the loss of DPA is also shown.

As can be seen the enrichment of the spore-forming nutrient medium in Ca induced increased thermoresistance and dipicolinic acid retention capacity in the spores. Both the DPA and Ca content of the spores obtained on medium rich in Ca was substantially higher than in spores formed on media of low Ca content. The exudation of dipicolinic acid followed nearly linear kinetics as a function of thermal treatment time.

2.2. Ion exchange chemical treatment of spores

In another series of experiments the aqueous suspension of the chemically treated spores as described in para. 1.6, was exposed to an activating heat treatment of 10 min at 80°C and the thermoresistance of the spores was investigated upon treatment at 90°C. The results were compared to those obtained

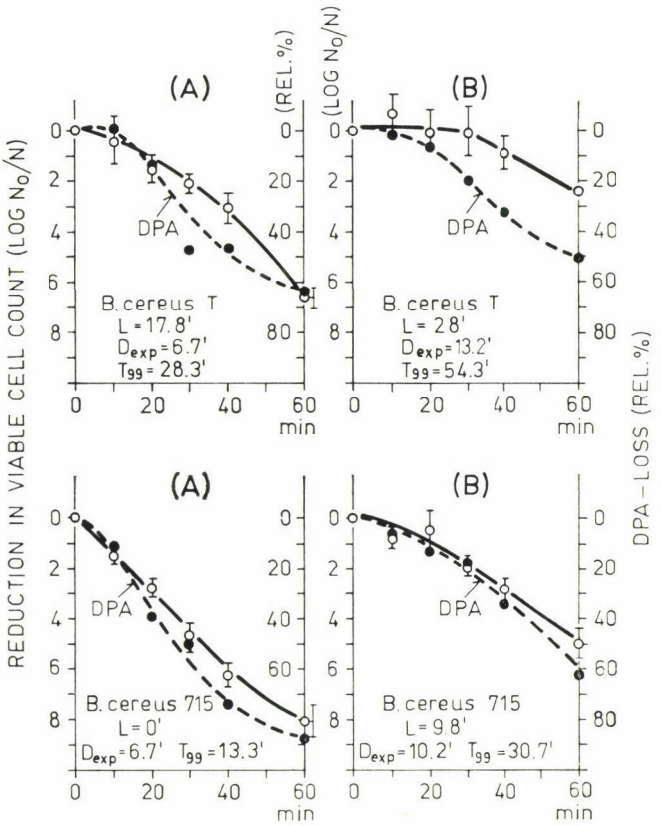

Fig. 1. Heat resistance of the spores of two *Bacillus cereus* strains at 90 °C and the loss of DPA in the course of the treatment as a function of Ca concentration in the medium used for spore formation. (A) Nutrient medium of 3.7 m*M* Ca concentration. (B) Nutrient medium of 24.5 m*M* Ca concentration. L – Length of the shoulder of the survivor curve (the value projected on the abscissa of the intersection of the tangent drawn to the steepest section of the survival curve and of the origin of the initial survival level). D_{exp} = decimal reduction time in the exponential section of the death curve; T_{99} = heat treatment time required to the 99% reduction of viable cell count; N_0 = initial viable cell count (ml^{-1}); N = residual viable cell count (ml^{-1})

with native spores not given preliminary chemical treatment. Survivor curves are shown in Fig. 2.

As can be seen in this figure, the survivor curves of the native spores had an exponential character up to a reduction in the viable cell count of 5 to 6 log cycles, the survival curves tailed in the region of low viable cell counts. It can be seen further that, in practice, the thermoresistance of the spores exposed to acid treatment (H^+ form) corresponded to the original thermoresistance of the spores of both strains, shown in spores formed on the given nutrient medium. Thus, the native spores were in this case mostly of H^+ form. The resistant "tail-fraction" was less evident in the acid-treated population. The thermoresistance of spores surviving Ca-acetate treatment and heat activation,

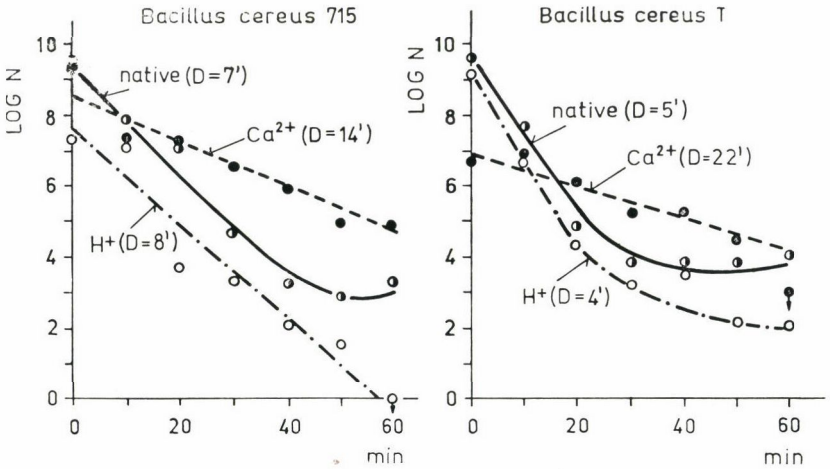

Fig. 2. Changes in the heat resistance of the spores of two *Bacillus cereus* strains (heat treatment at 90°C) subsequent to chemical treatment inducing ion exchange. N = viable cell count (ml^{-1}); H^+ = spores incubated prior to thermal destruction in HCl solution of pH 3.5 at 60°C for 1 h then at 25°C for 3 h (after treatment the pH of the spore suspensions was readjusted to pH 6.5 with NaOH. The suspension was then centrifuged and washed in distilled water 3 times. Prior to the thermal destruction test the spores were heat-activated at 80°C for 10 min). Ca^{2+} = spores incubated prior to thermal destruction in a 0.02 *M* Ca-acetate solution adjusted to pH 8.0 at 60°C overnight. (After the Ca-treatment the spore suspensons were 3 times centrifuged and washed in distilled water and exposed to heat-activation at 80°C for 10 min prior to thermal destruction.) Native spores not given chemical treatment to induce ion exchange and heat-activated at 80°C for 10 min. D = decimal reduction time calculated from the steep section of the survival curve

and probably transformed into Ca^{2+} form, was substantially higher. As can be seen, however, during the ion exchange treatments a more or less extensive reduction occurred in viable cell counts and this was particularly substantial with the *Bacillus cereus* T strain, after treatment with Ca. Complementary investigations have shown that this ion exchange treatment induced in a fraction of the spores a germination-like change (reduction of the refractive index, exudation of DPA). This is shown in Fig. 3.

Cells, thus converted, did not survive incubation at 60°C and heat-activation for 10 min at 80°C. The relative number of "darkened" spores and the amount, of DPA, exudating from the cells in the course of germination and thermal death during ion exchange treatment, were proportional to the reduction of viable cell count.

In repeating the experiment the Ca treatment was carried out at 30°C for 24 hours, instead of at 60°C, germination appeared more extensive, while the fraction of spores not losing dormancy showed a highly increased thermoresistance.

During the long cold storage period of H^+ and Ca^{2+} form spore populations, subsequent to ion exchange treatment, a slow reduction in the viable

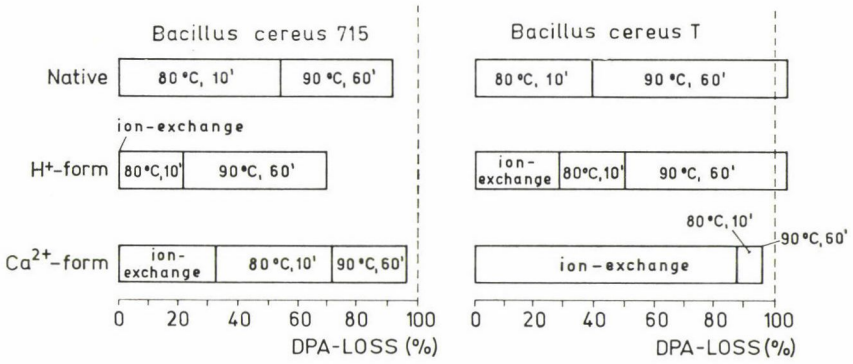

Fig. 3. DPA loss in the spores of *Bacillus cereus* 715 and *Bacillus cereus* T strains, resp., during the preliminary chemical treatment, the heat-activation at 80°C for 10 min, and the lethal treatment at 90°C for 10 min. For ion exchange treatment see the legend to Fig. 2. The DPA-loss was obtained by determining the dipicolinic acid concentration in the supernatants. The total DPA content was understood to be the amount of dipicolinic acid obtained by heating the aqueous spore suspension in an autoclave at 1 atm gauge pressure, for 15 min

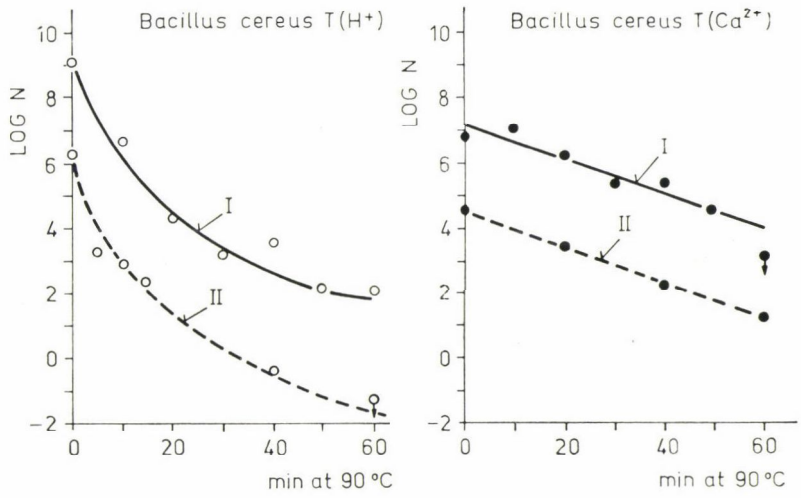

Fig. 4. Survival of spores given ion exchange treatment, after thermal destruction at 90°C and during 6 months storage at refrigerator temperature in an aqueous suspension. (I) Survival curve immediately after chemical pretreatment. (II) Survival curve after chemical treatment and 6 months cold storage. For symbols (H^+) and (Ca^{2+}) see legend to Fig. 2

cell count occurred, however, the thermoresistance of the surviving populations remained practically unchanged.

The thermoresistance of spores obtained from a nutrient rich in Ca was essentially the same as that of Ca^{2+} form spores gained by ion exchange (Fig. 5).

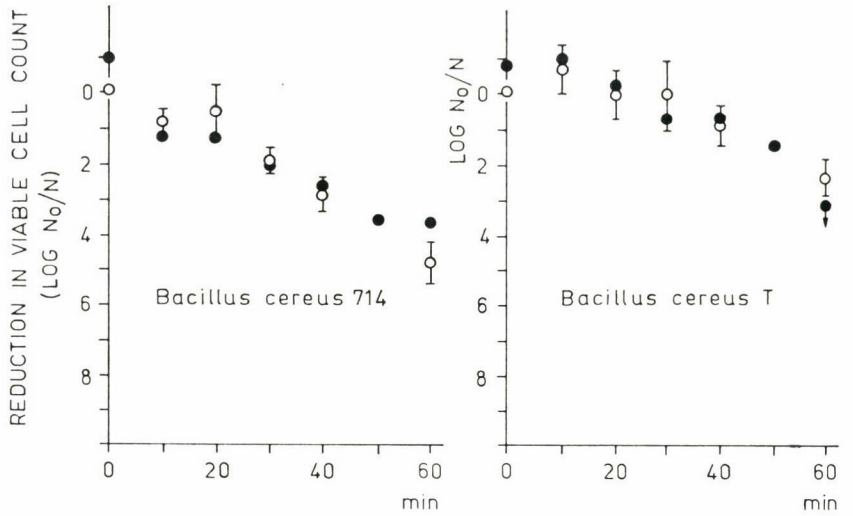

Fig. 5. Comparison of the thermal destruction at 90°C of spores ○ obtained on a nutrient medium rich in Ca (24.5 mM) with the spores ● obtained on a nutrient medium of low Ca content (3.7 mM) then converted as described in para. 1.6 to their Ca^{2+} form

3. Conclusions

It seems probable that the effect of the ion exchange treatment on the thermoresistance of ungerminated spores is related to the change in the thermostability of spore membranes (FITZ-JAMES, 1971). The spore germination initiating effect of calcium treatment seems to prove the "expanded cortex" hypothesis of GOULD and DRING (1974a). The pressure exerted by the expanded cortex on the spore core is assumed to ensure dormancy and presumably dehydrated state of the spore protoplast. The entering of cations and basic molecules or salts at a high concentration into the electro-negative cortex may cause the collapse of the expanded structure and thereby the hydration of the spore protoplast (GOULD & DRING, 1974b). It seems that the Ca-acetate treatment had the same effect upon the majority of the spores in these experiments, while those in which for some reason or other the peptidoglycan in the cortex did not react with the Ca in the medium, showed an increased heat stability.

Though, unlike in the experiments of ALDERTON and SNELL (1969a) with *B. stearothermophilus*, in the experience of the authors, acidic treatment sensitized the majority of spores only slightly, it seemed to reduce the thermoresistance of the small number of highly resistant cells. By the application of similar treatment to some foods prior to canning, the sporocidal effect of heat treatment might possibly be increased. However, this has to be proven in canning experiments.

The great difference in the heat resistance of H^+ form and Ca^{2+} form spores shows that it is not expedient to leave the resistance of the cultures of spore formers, used in the canning industry as test organisms, to chance conditions of nutrient medium and incubation. In this case the heterogeneity of resistance may be extensive and the spores show only a fraction of their potential heat resistance in the thermal death experiments.

*

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CONTENTS

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DETERMINATION OF PECTINMETHYLESTERASE, POLYGALACTURONASE AND PECTIC SUBSTANCES IN SOME FRUITS AND VEGETABLES

PART II. – STUDY INTO THE PECTOLYTIC TISSUE ENZYMES OF *JONATHAN* APPLES AND SOME OTHER FRUITS AND VEGETABLES

M. POLACSEK-RÁCZ and K. POZSÁR-HAJNAL

(Received January 27, 1975)

A method has been developed to determine the activity of low concentrations of polygalacturonase (PG) and pectinmethylesterase (PME) occurring in fruit extracts. A more detailed study was made into the pectolytic tissue enzymes of *Jonathan* apples and their changes during ripening and storage of the fruit. The PG and PME activities of some fruits and vegetables were determined. These were: strawberries, raspberries, red currants, gooseberries, apricots, peaches, plums, pears, early red radish, carrots and glass-house cucumbers.

The results may be summarized as follows.

1. Of the horticultural produce studied the PME activity of *Jonathan* apples, similarly to that of tomatoes, could be continuously measured by titration. The amount of galacturonic acid liberated from the substrate during the reaction was linearly correlated with reaction time (Fig. 1).

2. PME activity was highest in the apple extract prepared by adding 5% NaCl to the homogenate, while NaCl contents of 7.5 or 10% inhibited the enzyme (Fig. 3). During serial activity measurements the salt concentration in the reaction mixture exceeded 1.5%.

3. The PG activity of the apple extract exhibited a linear correlation with the square root of reaction time and also with the logarithm of the amount of apple extract. While the former observation corresponded with that gained with tomato extracts, the latter observation was in contrast with it (Fig. 2).

4. During storage of the apple extract at 5 °C the PME activity substantially decreased (in 1–2 days by 41% on the average, Fig. 4) and the PG activity increased as a function of storage time (Fig. 5).

5. To measure the PG activity, 50 °C proved more suitable than the lower temperatures. However, in the case of plums, strawberries and red currants 40 °C was the most suitable temperature. This is probably the consequence of the difference in the temperature-optimum or the heat sensitivity of the enzymes (Table 2).

6. As with tomatoes, PME and PG activities increased in all samples in the course of ripening (Tables 1 and 2).

7. Both the PME and PG activities of the samples examined decreased upon freezing or storage at −20 °C, just as in the case of tomatoes (Table 1).

In the first part of this series we gave an account of the experiments carried out with the tissue enzymes of tomatoes. Two enzymes, polygalacturonase (PG) and pectinmethylesterase (PME) play the most important role in the degradation of pectin. The temperature and pH optima of the two enzyme components and the dependence of their activity on reaction time and on enzyme concentration, resp., were investigated. The results of these experiments have been used in the determination of enzyme activity in other fruits and vegetables, though it is not certain that the parameters found op-

timal in the case of tomatoes are optimal also for the enzymes of other plants. In agreement with earlier studies (VAS *et al.*, 1968), the concentration of these enzymes was lower in the other fruits studied. In some produce they were hardly detectable, thus these produces are less suitable for the study of the enzymes.

Of the fruits of lower enzyme concentration the pectolytic tissue enzymes of apples were studied and the changes occurring during ripening and storage were followed up. The pectolytic enzyme activity of some fruits and vegetables of shorter season was also established. The list of produce studied is as follows: apples (*Jonathan*), strawberries, raspberries, red currants, gooseberries, apricots, peaches, plums, pears, early red radish, carrots and hothouse cucumbers.

1. Materials and methods

1.1. Fruits

The fruits and vegetables tested were bought on the market. The activity was determined in most cases in the fresh produce, in some cases after storage at 5 °C or at −20 °C.

1.2. Extraction of the enzymes from the plant tissues; substrates used for activity measurement

The same pectin preparations were used as the substrate as in the first part of this series [*Obipektin "Violettband"*, OBIPEKTIN A. G., Bischofszell, Switzerland, apple pectin of 30–36% esterification, low-ester pectin (0) and *Pomosin*, POMOSIN-WERKE GMBH, FRG, apple pectin of about 70% esterification, high-ester pectin (P); POZSÁR-HAJNAL & POLACSEK-RÁCZ, 1975].

The enzymes were extracted with the same salt solutions and salt-containing buffer solutions as described in the first part of this study. The phosphate buffer of pH 7.5 containing 5% NaCl was in every case more suitable for the extraction of both PME and PG than the aqueous solution of 5% NaCl, when applied in a proportion of 1 : 1 (w/w) to the tomato homogenate. However, since, in the case of the products of lower enzyme concentration, substantially more extract had to be added to the substrate, the buffer present in high concentration inhibited or made impossible the measurement of activity. Thus, only aqueous salt solution was used for extraction (5% NaCl solution, 1 : 1, w/w). In the case of apples and cucumbers the product was grated and 100 g of the materials were mixed with 2.5 or 5 g of NaCl. The enzyme activity was measured in the juice pressed by hand from this mixture. With 2.5 or 5 g NaCl added, 100 g grated apple yielded 66 ± 4.3 ml

and 72 ± 15.3 ml juice, resp. From 100 g peaches 145 ± 24 ml of extract were obtained with the NaCl solution. In the case of 2.5 or 5% added NaCl, the salt contents of the extracts were 3.8 ± 0.2% and 6.9 ± 1.2%, resp.

In the case of apples the effect of the method of homogenization on the activity of the extract was also studied. The activity was measured in the extracts obtained from grated or homogenized apples by the addition of NaCl.

With some of the fruits, for instance with apples, the effect of storage of the extract at refrigerator temperature was also investigated.

1.3. Determination of PME activity

The PME activity in apples and the other fruits studied was lower than that in tomatoes, therefore the method described in the first part of this series of study had to be modified; 60 ml of a 0.5% pectin solution were used as the substrate. To study the activity in apple extract the low-ester pectin, with the other fruits and vegetables the high-ester pectin was used. This is shown in Table 2. Depending on their activity, 10 to 40 ml of the extracts were added to the substrate. The final pectin concentration in the reaction mixture was about 0.3–0.4%. The error caused by the reduction of the pectin content in the reaction mixture was negligible in the case of extracts of low activity (Vas *et al.*, 1967; Sanner *et al.*, 1972). Measurements were carried out at 30 °C in the mixtures with their pH adjusted to 7.5, as was found optimal for tomato extracts. The amount of carboxyl groups liberated during hydrolysis was determined by titration with 0.1 *N* NaOH. Because of the lower activity, in the first 10–30 min of the reaction the alkali required to keep the pH at constant level was read every one or two minutes. Thus, related to the method as described for tomatoes, both the amount of extract applied and the reaction time had to be increased.

In case of fruits of very low activity (gooseberries, strawberries, red currants, apricots, pears) the reaction was continued for 2 hours then stopped by rapid cooling of the mixture and the concentration was given in U g^{-1}. The extract used for comparison was inactivated by boiling for 30 min.

1.4. PG activity measurement

In the fruits and vegetables tested both the PME and the PG activities were lower than in tomatoes. Therefore the PG activity could not be measured by the method described in the first part of this study, based on continuous viscometry. The method developed by Zetelaki-Horváth (1972) for the measurement of PG activity in mould fermentation broths was adapted. The principle of the method is as follows: a 0.25% low-ester pectin solution is used as substrate. To 10 ml substrate 1–5 ml fruit extract are added, depend-

ing on the latter's activity. The mixture is then incubated for 0.5–5 hours at 40 or 50 °C, depending on the kind of fruit. Samples are taken every half hour. The activity of the enzyme is stopped by rapid cooling and 7 ml are transferred to a viscometer and the viscosity measured in a water-bath of 25 °C.

The blank is prepared in a similar way, only the fruit extract added to the substrate is inactivated by boiling. In calculating viscosity, the delivery time of the substrate is related, instead of to the flowtime of water, to the delivery time of a mixture of 10 ml water and the same amount of fruit extract as used in the sample. Degree of degradation (D, %) is calculated from the time of delivery, in seconds, of the aqueous fruit extract (blank) and of the samples, by means of the correlation described in the first part of this series (POZSÁR-HAJNAL & POLACSEK-RÁCZ, 1975).

The PG enzyme content of the fruit extracts was characterized by the "50% specific pectolytic activity" value (SPA_{50}). Since the viscosity of the mixture was not measured continuously, the enzyme quantity required to reach, under the experimental conditions, 50% D in 1 hour was considered as unit activity. In this context SPA_{50} is the number of ml of the 0.25% pectin solution degraded, under the experimental conditions given, to 50% of its original viscosity in 1 hour, by the extract obtained from 1 g of fruit.

The pectin substrate was used initially as a 0.25% aqueous solution of pH 3.6–3.8, but later on, as a result of the experiments carried out with tomatoes, its pH was adjusted to the pH optimum of tomatoes, 4.5. Thus a four-fold PG activity could be measured in the extracts and the 50% D could be attained with fruits where, because of low enzyme activity, this was not possible with an aqueous substrate.

2. Results

2.1. Study of the PME and PG activities of Jonathan apples

2.1.1. Measurement of the PME and PG activities in apple extracts. PME activity was measured, as described in para. 1.3, in a low-ester substrate of pH 7.5. The amount of galacturonic acid (G, μmol) liberated by 1 ml apple extract, showed a linear change as a function of reaction time. This is illustrated in Fig. 1.

The PG activity was determined in the 0.25% aqueous substrate after incubation at 50 °C. The D value as a function of reaction time and the amount of the enzyme-containing extract, is shown in Fig. 2.

Similarly to tomatoes (POZSÁR-HAJNAL & POLACSEK-RÁCZ, 1975) the PG activity of apple extracts up to 80% D showed a linear correlation with the square root of reaction time. Between 40 and 80% D, the corre-

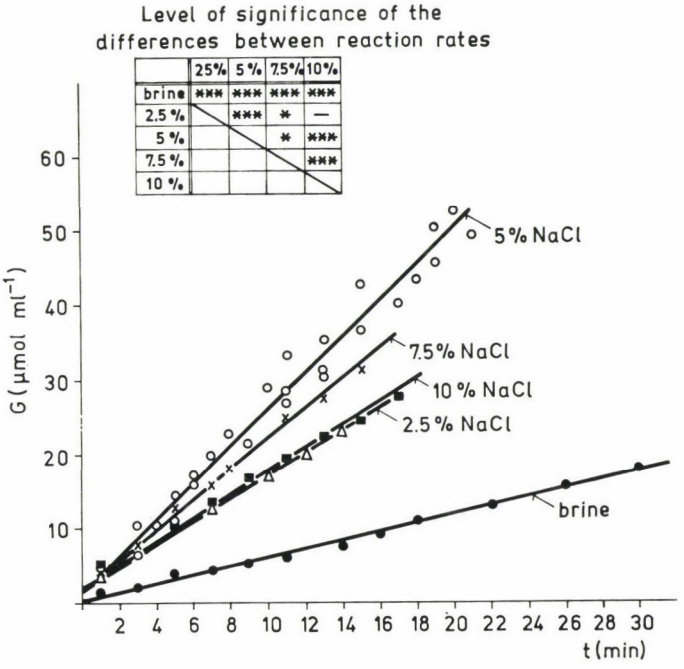

Fig. 1. PME activity in variously prepared apple extracts. Amount of galacturonic acid liberated by 1 ml apple extract (G, μmol ml^{-1}) as a function of reaction time (t). Substrate: 60 ml 0.5% low-ester pectin solution adjusted to pH 7.5; enzyme solution: *Jonathan* apple extract, 15–30 ml

Equations of the regression lines:

Extract prepared with brine (5% NaCl solution; 1 part to 1 part homogenate):	$G = 0.58t + 0.25$; $r = 0.997$
Extract prepared with 2.5% solid NaCl:	$G = 1.58t + 1.30$; $r = 0.997$
Extract with 5% solid NaCl:	$G = 2.44t + 1.36$; $r = 0.989$
Extract with 7.5% solid NaCl:	$G = 2.04t + 1.55$; $r = 0.996$
Extract with 10% solid NaCl:	$G = 1.55t + 2.10$; $r = 0.995$

Level of significance of the differences in reaction rate values: *** = difference significant at the 99.9% probability level; * = difference significant at the 95% probability level; – = difference non-significant (P < 95%)

lation between PG activity and the logarith mof apple extract concentration, was linear. Since the correlation between extract concentration and D was not linear, the amount of extract required to reach 50% D was determined by graphical interpolation.

2.1.2. Activity as affected by the method of extract preparation. It is difficult to gain tissue enzymes from apples partly because they are present in low concentration and partly because apple is not particularly juicy. Due to the low enzyme concentration the activity was measurable only in the juice obtained by direct salting-in of the fruit homogenate. The PME activity of apple extracts as affected by NaCl concentration is shown in Fig. 3.

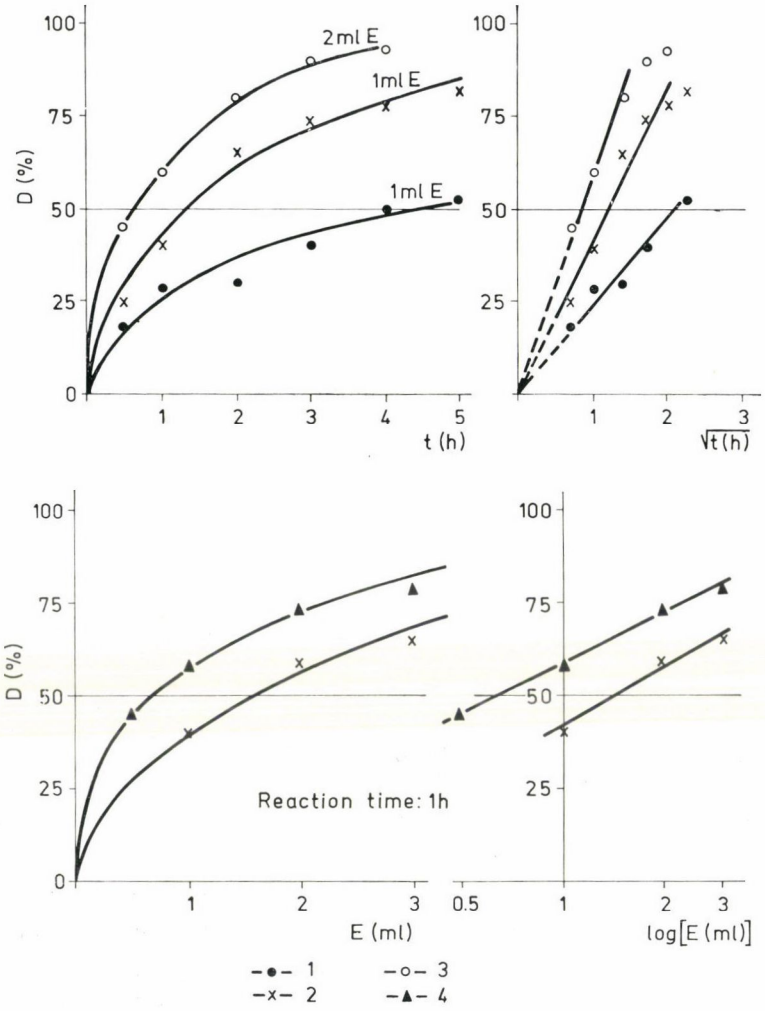

Fig. 2. PG activity in apple extracts (D) as a function of reaction time (t) and enzyme concentration. Substrate: 10 ml of 0.25% low-ester pectin solution; enzyme solution: ml apple extract prepared with 2.5% solid NaCl; total volume of the reaction mixture: 15 ml; incubation at 50 °C; **1**: stored for 4 days, apple extract No. 2; **2**: stored for 6 days, apple extract No. 2; **3**: stored for 7 days at 5 °C, apple extract No. 6; **4**: stored for 8 days at 5 °C, apple extract No. 6

The Figure illustrates the activity of the extract, obtained by pressing from 1 g apple, as a function of the amount of NaCl added (Fig. 3A) in preparing the extract and the calculated value of the NaCl content of the reaction mixture, resp. (Fig. 3B). As can be seen the PME activity obtained with 5% NaCl added is nearly the double of the activity with 2.5% NaCl, while 7.5 or 10% salt contents were of inhibitory effect. Extracts were prepared also from all the samples with 2.5% salt, because PG activity could be measured only in that case.

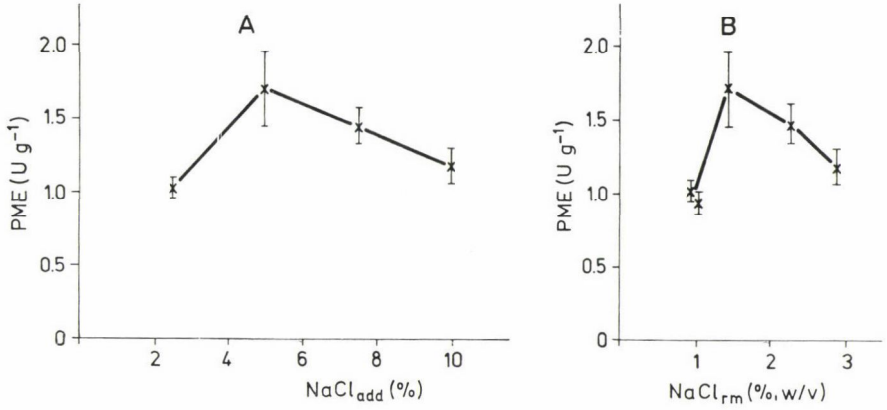

Fig. 3. PME activity (U) in apple extracts as affected by NaCl concentration. (A) Activity in 1 g apple as a function of added NaCl ($NaCl_{add}$, g NaCl per 100 g fruit) used for extraction. (B) Activity in 1 g apple as a function of NaCl content of the reaction mixture ($NaCl_{rm}$). For conditions of measurement see legend to Fig. 1. I = standard deviation

PME activity was studied in the homogenate containing NaCl, too. In this case 10 g of the above pulp were suspended with 20 ml water and added to the substrate. The measured value of activity was converted into the value belonging to 1 g apple. It was found that only half of the activity of the pulp could be recovered in the juice with 5% added NaCl, while only 21% with 2.5% NaCl (Fig. 4A, columns 1, 2, 3). However, to wash out the pulp was not expedient; because of the low total activity no PME could be measured in the diluted juice by the method applied.

Two methods were used in preparing apple extract: *1.* the grated apple was mixed with NaCl and stored at 5 °C in the refrigerator overnight; next day it was pressed through a muslin cloth; *2.* the grated apple was given a short homogenation with added NaCl, stored as before and then pressed for juice. The results have shown the latter method, *i. e.* homogenation after grating, more satisfactory from the point of view of both PME and PG extraction. PME activity in the juice thus obtained was about 15% higher (Fig. 4B, column 7) and PG activity about 33% higher than in the juice obtained from apples only grated. These results are summarized in Fig. 4 and Table 1.

2.1.3. Enzyme activity of apples and apple extracts as affected by storage. The effect of storage at 5 °C on the activity of apple extracts was studied. A difference was found as regards the effect of PME and PG activity, resp. As a result of 1–2 days' storage the PME activity decreased by about 41% (Fig. 4, column 6) while the PG acitvity increased as a function of storage time. The change in PG activity as a function of storage time in the refrigerator is illustrated in Fig. 5 and Table 1.

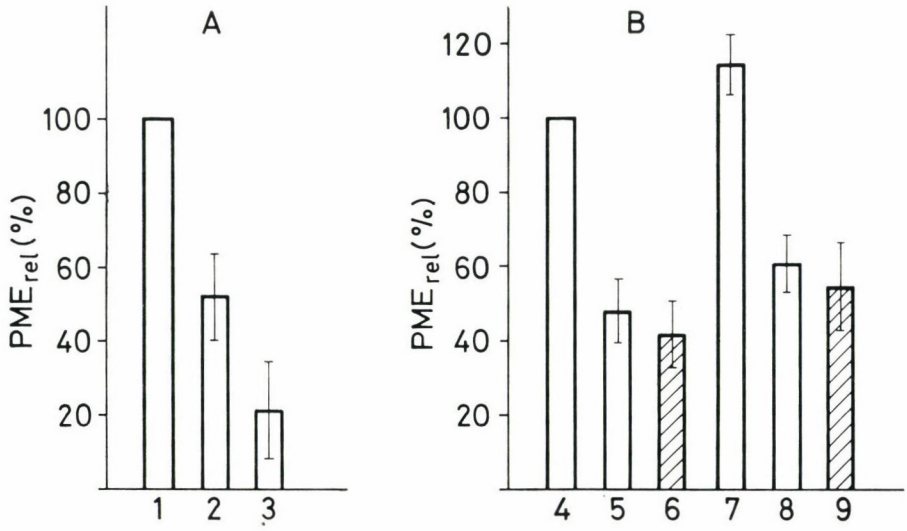

Fig. 4. Relative PME activity (PME_{rel}) in apple extracts as affected by the method of preparation. (A) Taking the total PME activity measured in 1 g apple pulp as 100%. (B) Taking the total PME activity measured in 1 g grated apple obtained by the addition of 5% solid NaCl as 100%. **1**: In the pulp; **2**: in the extract obtained with 5% solid NaCl; **3**: in the extract obtained with 2.5% solid NaCl; **4**: in extract obtained with 5% solid NaCl added to grated apple; **5**: in grated apple extract obtained with 2.5% solid NaCl; **6**: in juice gained from grated apple containing 5% solid NaCl after 2 days storage at 5 °C; **7**: in extract from grated, subsequently homogenized apple obtained with 5% solid NaCl; **8**: in grated, subsequently homogenized apple extract obtained with 2.5% solid NaCl; **9**: in grated, subsequently homogenized apple extract obtained with 5% solid NaCl after 2 days storage at 5 °C. Columns 7, 8 and 9 represent the averages of 2 measurements and their standard deviations. I = standard deviation

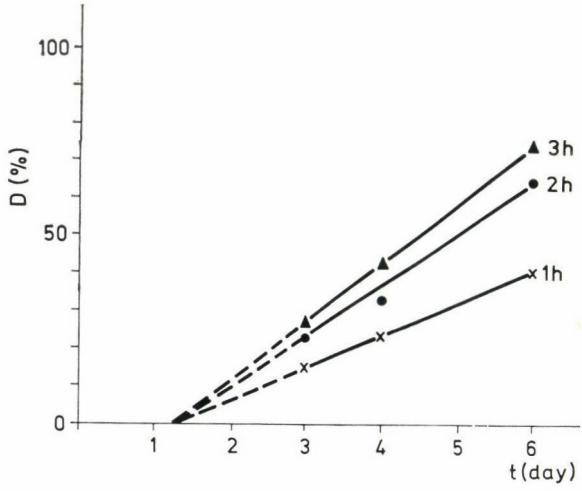

Fig. 5. PG activity (D) in apple extracts as affected by storage time (t) at 5 °C. Substrate: 10 ml low-ester pectin solution of a concentration of 0.25%; enzyme solution: 1 ml apple extract of 2.5% NaCl concentration, mark 2; reaction time: 1, 2 and 3 hours (h); temperature: 50 °C

Table 1

PME and PG activity in Jonathan apples as a function of date of analysis, storage temperature and period of the apple, method of preparation and the storage period of the extract, resp.

Date of measurement	No. of the sample	Method of preparation		Storage period of the extract at 5 °C (days)	Enzyme concentration	
		Method of comminution	NaCl** %		PG (SPA_{50}, ml $h^{-1}g^{-1}$)	PME (μmol $min^{-1}g^{-1}$)
19. 01. 1973	1	r	2.5	0	Ø	1.0
		r	5.0	0	Ø	1.8
		r	5.0	2	Ø	0.7
		r	2.5	4	0.89	—
		r	2.5	6	2.16	—
01. 02. 1973	2	r	2.5	0	+	0.35
		h	2.5	0	+	0.5
		r	5.0	0	—	0.8
		h	5.0	0	—	0.9
		r	5.0	2	—	0.35
		h	5.0	2	—	0.4
		r	2.5	2	1.07	—
		h	2.5	2	1.45	—
		r	2.5	3	1.27	—
		h	2.5	3	1.67	—
		h	2.5	4	1.79	—
		h	2.5	7	2.18	—
		h	2.5	8	2.22	—
14. 02. 1973	2/a	r	2.5	0	3.00	0.42
15. 02. 1973	3	h	2.5	0	5.5	0.82 2.25*
		h	5.0	0	—	2.1 4.1*
		h	2.5	2	7.1	—
		h	2.5	5	10.2	—
30. 03. 1973	3/f	h	2.5	0	2.5	0.42
16. 04. 1973	4	h	2.5	0	Ø	Ø
		h	2.5	4	Ø	—
30. 10. 1973	5	h	2.5	0	—	0.2
		h	2.5	6	0.5	—
	6	h	2.5	0	—	0.15
		h	2.5	6	1.0	—
	7	h	2.5	0	—	0.05
		h	2.5	6	0.47	—

Symbols: a = apple from the batch marked No.2 stored for 2 weeks at 30 °C
f = apple from the batch marked No.3 stored for 6 weeks at −20 °C
r = extract gained from grated apple
h = extract of grated and subsequently homogenized apple containing NaCl
Ø = activity low, not measurable
— = activity not measured
* = PME activity measured in the pulp
+ = increase in viscosity
** = solid NaCl added to the apple homogenate

Serial numbers represent apple batches bought at various dates (Nos. 1–7)

As can be seen, no PG activity could be demonstrated in the apple extract stored for only 1 or 2 days. In these samples the viscosity increased during incubation. Decrease in the viscosity of the apple juice kept in the refrigerator was observed only after storage for 3 to 8 days and the increase in activity with storage time was linear.

In apples stored at 30 °C not only the PG activity increased, but to some extent the PME activity as well. The slight increase of the latter amounted to about 14%. Numerical values are given in Table 1.

Enzyme activity as affected by storage at −20 °C was also studied. Both the PG and PME activities of samples obtained from apples after frozen storage at −20 °C were lower than prior to freezing. After a storage period of six weeks the loss of activity amounted to 45% for both enzymes.

The effect of ripeness of the apple batches upon their pectolytic activity was also studied. Results are shown in Table 1. As can be seen in the table the enzyme activity increased during ripening. In apples investigated in October, soon after harvesting, hardly any activity could be detected (samples marked 5, 6 and 7). In apples bought in January or February, after commercial storage, enzyme activity was well measurable (samples marked 1 and 2). The activity in the apples stored after harvesting in the cold store of the Institute and analysed the following February (sample marked 3) was even higher. In apples obtained from the primary producer in April, at the end of the storage period (sample marked 4), neither PG nor PME activity could be detected.

2.2. Tissue enzymes in some other fruits and vegetables

A preliminary study of tissue enzymes in fruits and vegetables as listed above was carried out. Partly because of the short seasonal character and partly because of the low enzyme content of these products only one or two batches each were investigated. Results are summarized in Table 2. In most cases a brine of 2.5% NaCl was used for extraction. If otherwise, pertinent data are given in the table.

By the continuous titration method, used with apples, of the fruits listed in the table PME activity could be demonstrated in peaches, raspberries and plums, of the vegetables in early red radish and cucumbers. In the last two cases 0.5% solution of high-ester substrate and a large amount of extract were used. The activity in gooseberries, apricots and pears could be detected only after a degradation period of two hours. In the extract of red currants and strawberries PME activity could not be detected at all.

PG activity was measured on a substrate of low esterification with pH set at 4.5. The reaction mixture was incubated, depending on the fruit, at 40 or 50 °C and the SPA_{50} value was calculated from the result obtained at the temperature more favourable for the given fruit. Of the fruits studied the PG activity in strawberries, red currants and plums was higher at 40 °C, while the rest showed a higher activity at 50 °C.

The PME and PG activities in peaches, plums and currants were studied in the fresh and in the quick frozen fruit. The enzyme activity, as in the case of apples, was found substantially higher in the fresh than in the frozen fruit.

Of the vegetables investigated the highest PG and PME activities were observed in early red radish. Since cucumbers are of high moisture content, instead of the aqueous extract, the juice pressed from the grated and salted flesh was used for activity measurement. The saline extract of carrots was not suitable for PG activity measurement. If a small amount of extract was added to the substrate the activity was not measurable. On adding a larger amount, the reaction mixture formed a gel. Gel formation occurred in a substrate of high esterification, thus PME activity, too, could be measured on a low-ester substrate, only.

The highest PG and PME activity values measured in this study in various fruits and vegetables are compared to those obtained for tomatoes in Fig. 6.

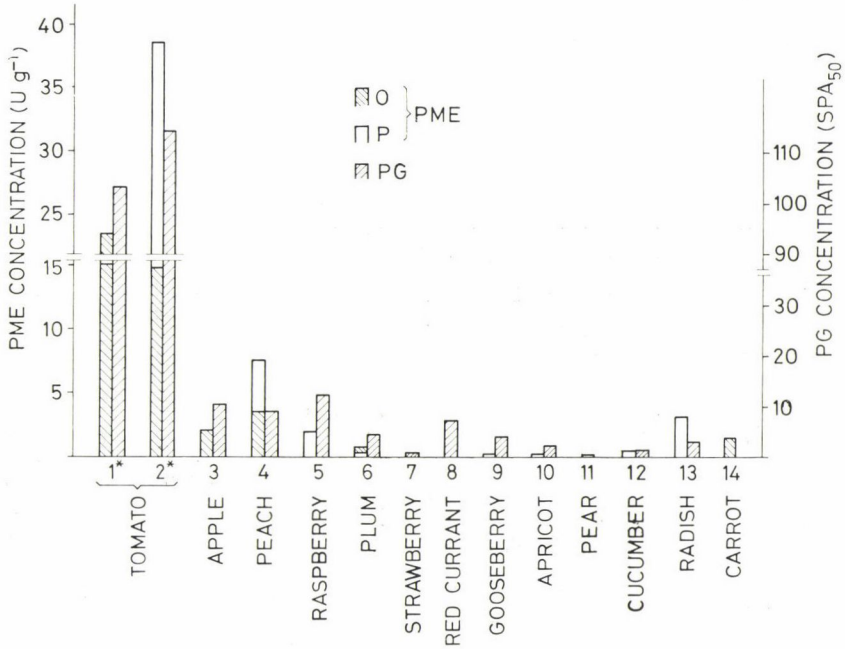

Fig. 6. PME and PG activity in the horticultural produce investigated. The maximum concentration value is given for each produce. 1*. Tomatoes (1 October, 1972); 2*. Tomatoes (2 October, 1973); 3. *Jonathan* apples (15 February, 1973); 4. Peaches (September, 1973); 5. Raspberries (PME: July, 1972; PG: July, 1973); 6. Plums (13 April, 1973); 7. Strawberries (18 June, 1973); 8. Red currants (4 July, 1973); 9. Gooseberries (11 July, 1973); 10. Apricots (25 July, 1973); 11. Pear (25 July, 1973); 12. Cucumber (7 May, 1973); 13. Early red radish (7 May, 1973); 14. Carrots (21 May, 1973). * = Data taken from the first part of this series (POZSÁR-HAJNAL & POLACSEK-RÁCZ, 1975); O = PME concentration measured on a low-ester pectin substrate; P = PME concentration measured on a high-ester pectin substrate

Table 2

Pectolytic activity in the tissues of some fruits and vegetables

Produce	Date of measurement	Condition of the produce tested	Additive used with homogenization		Activity in 1 g of produce			
					PME		PG	
			Material	NaCl content* (%)	Substrate	U g^{-1}**	Temperature (°C)	SPA_{50}+
Peach	18. 12. 1972	quick-frozen in the laboratory	NaCl solution	5.0	O	1.60	50	1.25
	07. 1973	fresh	NaCl solution	5.0	O	Ø	50	3.4
	09. 1973	fresh	NaCl solution	5.0	O	3.61	50	8.9
	09. 1973	fresh	NaCl solution	5.0	P	7.59		—
Paspberry	07. 1972	fresh	distilled water	0		—	50	10.5
	07. 1972	fresh	buffer containing NaCl	5.0		—	50	12.2
	26. 06. 1973	fresh	NaCl solution	1.25	O	1.74		—
	18. 07. 1973	fresh	NaCl solution	2.5	P	2.00		—
	18. 07. 1973	fresh	distilled water	0	P	Ø		—
Plum	13. 04. 1973	quick-frozen in the laboratory	NaCl solution	5.0	O	0.82	40	4.5
	13. 04. 1973	quick-frozen in the laboratory	NaCl solution++	5.0	O	0.72		—
	13. 04. 1973	quick-frozen in the laboratory	NaCl solution	5.0	P	0.36		—
	26. 04. 1973	commercially quick-frozen	NaCl solution	5.0	O	0.40	40	Ø
	26. 04. 1973	commercially quick-frozen	NaCl solution++	5.0	O	0.32		—
	26. 04. 1973	commercially quick-frozen	NaCl solution	5.0	P	0.09		—
Strawberry	18. 06. 1973	fresh	NaCl solution	5.0	P	Ø	40	0.67
Red currant	05. 1973	commercially quick-frozen	NaCl solution	5.0		—	40	Ø
	18. 06. 1973	fresh	NaCl solution	5.0	P	Ø	40	1.75
	04. 07. 1973	fresh	NaCl solution	5.0	P	Ø	40	7.3

Gooseberry	04. 07. 1973	fresh	NaCl solution	5.0	P	Ø	50	2.7
	11. 07. 1973	fresh	NaCl solution	5.0	P	0.15	50	4.0
Apricot	25. 07. 1973	fresh	NaCl solution	5.0	P	0.17	50	2.34
Pear	25. 07. 1973	fresh	NaCl solution	5.0	P	0.08	50	
Cucumber	07. 05. 1973	hot-house	NaCl (solid, 2.5% added)		P	0.47	50	1.15
	07. 05.;1973	hot-house	NaCl (solid, 5.0% added)		P	0.32		—
Early red radish	07. 05. 1973	fresh	NaCl solution	5.0	P	3.21	50	3.0
Carrot	21. 04. 1973	fresh	NaCl solution	5.0	O	1.56	40	gel formation

Symbols:

* = liquid additives applied in a proportion of 1 : 1 (w/w) to the fruit homogenate
** = in general, activity was measured at 30 °C, with apricots and pears at 50 °C
\+ = activity measured on low-ester pectin substrate
++ = PME activity measured in the fruit pulp
0 = low-ester pectin solution
P = high-ester pectin solution
Ø = minimal activity, not measurable
— = activity not measured

It can be seen in the Figure that the enzyme concentrations found in tomatoes were higher by 1 or 2 orders of magnitude than the values for the other fruits and vegetables investigated. A similar difference in order of magnitude was reported by VAS and co-workers (1968). Apart from tomatoes the enzyme concentration in *Jonathan* apples, peaches, raspberries, plums and early red radish could be easily measured.

3. Conclusions

Of the fruits and vegetables investigated, apart from the tomatoes examined in the first part of this series, *Jonathan* apples are the most important and grown in the greatest quantity in Hungary. Therefore this was studied most extensively. Since the pectolytic activity in apples is low an extract of measurable enzyme activity was obtained only if the grated apple was homogenized with salt. The correlation between the PME activity and reaction time was found to be linear in the extract thus gained, just as in the case of tomatoes (Fig. 1). The PG activity, however, was in linear correlation with the square root of reaction time. The correlation between the PG activity and the concentration of apple extract was shown to be logarithmic in nature in the range of 40–80% D (Fig. 2).

To extract the highest PME activity addition of 5% solid NaCl to the apple homogenate was proven most satisfactory, while the PG activity could most favourably be measured in extracts prepared with 2.5% solid NaCl added. A 7.5 or 10% solid NaCl added to the fruit exerted an inhibitory effect on the measurement of PME activity (Fig. 3). Probably some NaCl is necessary to extract, by way of osmosis, the enzyme from the cells. However, a high NaCl concentration in the extract seems to inhibit the enzyme activity. A NaCl concentration above 1.5% in the reaction mixture had an inhibitory effect. These results are in agreement with the findings of VAS and co-workers (1967).

In plums, strawberries and red currants the highest PG activity value was obtained at 40 °C, while for all other fruits 50 °C was the optimum temperature (Table 2). The PG activity of tomatoes yielded the highest value at 50 °C, too (POZSÁR-HAJNAL & POLACSEK-RÁCZ, 1975). This depends probably on the temperature optimum and/or on the heat sensitivity of the enzyme in the given fruit.

Therefore it would be of interest to study the temperature optima and the heat sensitivities of the pectolytic enzymes of various fruit species and varieties.

It was found that storage of the apple extract at 5 °C, affected PG and PME activities in different ways. The PME activity decreased substantially

during storage (Fig. 4), while PG activity increased with time (Fig. 5). Similar results were found with tomatoes (POZSÁR-HAJNAL & POLACSEK-RÁCZ, 1975). This phenomenon is probably due to the breakdown during storage and after-ripening of some tissue material exerting an inhibitory effect on PG activity, possibly present in unripe apples. This assumption seems to be supported by the higher PG activities found in the fresh extracts obtained from apples, which had been allowed an after-ripening period at 30 °C, than in their extracts stored for 8 days in the refrigerator.

The effect of ripening upon the pectolytic activity in apple extract was also studied. Both PG and PME activities seemed to increase with ripening (Table 1). This result is in agreement with the experiences gained with tomatoes (POZSÁR-HAJNAL & POLACSEK-RÁCZ, 1975) as well as with data found in the literature (ROUSE *et al.*, 1962, 1964; NAGEL & PATTERSON, 1967; PRESSEY *et al.*, 1971). The PG activity was higher in ripe peaches, red currants and gooseberries than in the unripe fruits (Table 2).

Quick-freezing and storage at −20 °C were found to reduce PG and PME activity by about 45% as compared to that in the fresh fruit. These results are similar to those obtained with tomatoes (POZSÁR-HAJNAL & POLACSEK-RÁCZ, 1975).

The results obtained in this study are preliminary only, because they are based on the investigation of fruit batches of a single season. For some of the fruits the season is very short and methodological difficulties were caused by the low enzyme concentrations.

*

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BACTERIAL ASSIMILATION OF METHANE AS AFFECTED BY THE CO_2 CONTENT OF THE GAS ATMOSPHERE

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Experiments were carried out to utilize CO_2-containing natural gas for the production of high-protein biomass by microbiological methods. The effect of the CO_2 component on the methane assimilation of 3 bacterial cultures (1 pure culture and 2 mixed cultures) was studied. The change in the rate of gas assimilation was determined in methane–air mixtures containing 5, 10 or 15% CO_2, resp., and compared to the control mixture of 20% methane and 80% air.

In order to establish whether CO_2 is utilized or exerts a stimulating or inhibiting effect on methane assimilation, gas assimilation was studied in media containing intermediate products of methane assimilation and in gas mixtures of varied CO_2 content. Shake cultures were used to establish the growth of bacteria in metabolite containing nutrient medium with gas mixtures of different CO_2 contents.

The effect caused by changes in the CO_2 content of the 3 cultures differed. However, CO_2 concentrations above 5% exerted a detrimental effect as compared to the control.

CO_2 concentrations of/or below 5% exerted not simply a stimulatory effect but were incorporated with the help of the intermediary products.

The experiments have shown the incorporation of CO_2 to occur by way of methane metabolism. The incorporation of CO_2 seemed to have a favourable effect on the cultures of Strain H_{513} and Culture V, while it reduced the nucleic acid content in the case of Strain 5.

Thus, with the bacterial strain studied, the CO_2 concentration must not be increased above 5% at the cost of methane concentration.

The microbiological oxidation of methane occurs in several steps:

$$CH_4 \rightarrow CH_3\text{–}OH \rightarrow HCOH \rightarrow HCOOH \rightarrow CO_2$$

According to WHITTENBURY (1969), in multiplying cells, 80–90% of the formaldehyde is incorporated into cell carbon, while the rest is oxidized to acid and CO_2. BLEVINS and PERRY (1969) found that bacteria oxidized 60% of the hydrocarbons, while assimilating only 40%.

Several authors ascribe a stimulatory effect to CO_2, therefore they add it in concentrations of 5–10% to the initial gas mixture. HUTTON and ZOBELL (1949) found a CO_2 concentration of 0.03% sufficient for growth, but consider it expedient to add 5–10% to the initial gas mixture. They observed a slight reduction of methane assimilation at 20–30% CO_2 content. BEERSTECHER (1954) in his summary paper suggests the application of 5–10% CO_2. DWORKIN and FOSTER (1956) consider 0.3% to be the optimum CO_2 concentration.

SMIRNOVA (1971) found that, in the case of the strains studied, at the concentration of 5% CO_2 the lag period was substantially shorter and the biomass produced substantially larger than in an atmosphere not containing CO_2.

The observation of JIZUKA and co-workers (1966) points to this finding. They found methane assimilating microorganisms widely spread, however, a close correlation seemed to exist between the frequency of their occurrence in the given microbial flora and the CO_2 concentration in the ambient atmosphere.

SHEEHAN and JOHNSON (1971) applied 3% CO_2 in shake culture, 3–5% CO_2 in continuous fermentation and an outgoing gas mixture even of 55% CO_2 did not seem to inhibit growth.

Beside the observations of SMIRNOVA (1971) and STRAWINSKI and TORTORICH (1955) on the stimulating effect of CO_2 in the gas atmosphere, the work of SOROKIN (1956, 1957) related to autotrophy, or chemosynthesis is worth consideration. SOROKIN (1957) used ^{14}C tracer and found that the energy liberated during methane oxidation covers the energy requirement of CO_3^{2-} transformation into cell carbon in chemosynthesis.

The observations showing, on certain occasions, about 40–50% of the carbon content of cells grown on methane to originate from CO_2, seem to support SOROKIN's hypothesis on autotrophic assimilation.

According to ZAVARZIN (1964), however, about 60–80% of cell carbon is of organic origin and only a maximum of 40% of inorganic (CO_2) origin. The same observation was made with typically heterotrophic organisms. Presumably the incorporation of CO_2 occurs at C_2 compounds and this is a metabolite of methanol.

ZAVARZIN (1964) and SMIRNOVA (1971) investigated the amount of ATP formed upon the oxidation of 1 *M* CH_4 on the basis of the thermodynamic equation of microbiological methane oxidation and came to the unambiguous conclusion that methane assimilation is a heterotrophic process. Thus, the incorporation of CO_2, cannot be explained by autotrophic assimilation. Presumably, CO_2 becomes linked to the intermediate products of methane assimilation (ZAVARZIN, 1964) as a result of condensation reactions.

KIRIKOVA and co-workers (1972) found that the energy required to bind CO_2 may come from the oxidation of methane or formaldehyde. The degree of oxidation of the substrate bears upon the amount of CO_2 bound.

On exploring the possibilities of the industrial production of feed protein or protein-vitamin concentrates high hopes are attached to methane assimilating microorganisms, methane beeing the cheapest carbon source met with in large quantities. The majority of the natural gases found in Hungary are rich in CO_2. Percentages of 30–70%, or even 70–90% CO_2 are not infrequent. These circumstances induced us to investigate the effect of CO_2

concentration in gas mixtures on the utilization of methane by microorganisms.

1. Materials and methods

1.1. Bacterium cultures

Methane assimilating bacteria were isolated from soil samples taken in natural gas fields, from condensed water and gasolene samples separated at gas wells, from soil and water samples taken at leakages of natural gas lines and finally from the sterile cotton filter inserted in the pipeline for untreated natural gas. Strains were isolated by the streaking technique (HALÁSZ & SIMEK, 1968).

The mixed methane-assimilating cultures are of similar origin, as the selected strains, the difference being that selection of strains in the culture obtained by reinjection and propagation of the first culture enriched in a methane-air atmosphere, has not taken place.

The mixed cultures were marked V and S, resp. These cultures come from the gas field at Hajdúszoboszló (gas well No. 72). They proliferate beside methane on traditional pepton and glucose containing nutrient media. The most favourable nitrogen source for both cultures was NH_4Cl, the next being urea and then $(NH_4)_2SO_4$. KNO_3 did not prove a suitable nitrogen source for Culture V.

It was shown in the propagation experiments that the generation period of the mixed cultures was not more favourable than that of pure cultures. However, the cell concentrations achieved in the fermentation broth under equal conditions were substantially more favourable (0.8–1.0 g l^{-1} against 0.5 g l^{-1}).

The pure culture was marked Strain H_{513}. This was a bacterium strain isolated from the free water of 61 °C of gas well No. 17, Hajdúszoboszló. Its most favourable nitrogen source was $(NH_4)_2SO_4$. This strain is capable of utilizing not only methane, liquid and semi-solid hydrocarbons but glucose and pepton as well. For the measurement in the *Warburg* apparatus, a cell concentration of 10^9 ml^{-1}, for the propagation experiments a cell suspension of a concentration of 5.10^7 ml^{-1}, giving an optical density value, OD = 0.2, was used.

1.2. Nutrient medium

The nutrient medium was selected as shown in Table 1. It was not complemented with trace elements. The pH of the nutrient medium was adjusted to pH 7.2 and the change during fermentation was compensated by the addition of 0.1 *N* NaOH.

Table 1

Composition of the synthetic nutrient solutions

	g l^{-1}
$MgSO_4$	0.10
Na_2HPO_4	0.60
KH_2PO_4	0.14
NaCl	1.00
$(NH_4)_2SO_4$	0.66
Composition of the nutrient solution enriched with metabolites	
formic acid	0.46
methanol	0.32

To prepare metabolite-containing media, methanol, formaldehyde or formic acid were added to the above synthetic medium in quantities of equivalent carbon content.

1.3. Methane and air atmosphere

The methane–air mixture of the required composition was obtained by setting the flow rate of methane and air under controlled pressure, with the help of a gas mixing container (HALÁSZ, 1969).

The methane and air atmosphere in the fermenter was obtained by letting the gas mixture to be tested flow through for 15 min. In preliminary experiments a mixture of 20% CH_4 + 80% air was found to be the most favourable as a control atmosphere, therefore this was used in the course of these studies.

1.4. Mixtures of methane, carbon dioxide and air

Gas atmospheres of the following CO_2 concentrations were applied: 0, 5 and 10%.

The gas atmospheres were of the following composition:

CH_4 (%)	CO_2 (%)	air (%)
5	15	80
10	10	80
15	5	80
20	—	80

a). The CO_2 concentration was increased at the cost of the methane concentration in the gas mixture. The methane–air mixture for the given bacterium concentration is optimal at 10–20% methane concentration. The generation periods calculated for 3% CH_4 + 97% air and 20% CH_4 + 80% air

showed a difference of about 20% (HALÁSZ, 1972). If the presence of CO_2 exerted an inhibitory effect, the difference in the generation periods in a gas mixture of 5% CH_4 + 15% CO_2 + 80% air and the control would significantly exceed 20%;

b) if CO_2 is assimilated or chemically bound during fermentation, it serves as a carbon source;

c) on applying a methane–air mixture, at higher cell concentrations, both carbon and oxygen are present in limited quantities and in the usual laboratory fermentation techniques material transfer is a limiting factor, too. Under conditions limited by methane the yield coefficient for both carbon and oxygen is more favourable, than in the case of oxygen as the limiting factor.

The gas mixture was prepared by the same method as the methane–air mixture.

1.5. Fermentation temperature

The temperature applied for all the strains and every propagation method studied, was 37 °C.

1.6. Analytical methods to follow methane assimilation

1.6.1. Changes in the turbidity of the liquid phase. The growth of bacteria was followed by changes in the turbidity of the liquid phase (HALÁSZ & SIMEK, 1968) using an ORION-GYEM (Budapest) spectrophotometer at 450 nm. Throughout this study, E will denote optical density at optical path length of 1 cm, and Δ E the change in optical density due to growth.

1.6.2. Changes in the pressure of the gas phase. Since methane assimilation is accompanied by the reduction of the gas volume it may be followed by the measurement of gas pressure. Measurements were carried out in the *Warburg* apparatus, according to the method of UMBREIT and co-workers (1957) making 3 parallel measurements and replicates. The atmosphere required was ensured by bubbling through the appropriate gas mixture. The gas mixture was bubbled through the culture for 1 hour and subsequently the manometer was read at l-hour intervals. The change of pressure was calculated for μl CH_4 absorbed per initial viable cell count per hour.

Measurements were carried out with the control gas mixture (20% CH_4 + 80% air) and with 5, 10 and 15% CO_2, resp.

In applying the *Warburg* respirometer for measurement of the gas absorption rate the following considerations were taken into account. Experience has shown that, for proper registration at hourly intervals, 2 ml of bacterium suspension of $5 \cdot 10^8$ ml^{-1} density are necessary (HALÁSZ & SIMEK, 1968). At a cell density of this order gas absorption is a limiting process. The rate

of methane assimilation was shown in these experiments to be a function of the methane concentration in the gas atmosphere (HALÁSZ, 1969, 1972) and using a bacterium concentration of 10^8 ml^{-1} a gas mixture of 20% CH_4 and 80% air proved to be optimal.

1.7. Determination of the protein content of bacteria

The protein content of the biomass was determined with Folin-Ciocalteu reagent according to DÉVÉNYI and GERGELY (1968) and HILL and co-workers (1967). Serum albumin was used for comparison.

1.8. Nucleic acid determination in the biomass

Nucleic acid was extracted from the biomass according to *Schneider* (MUNRO & FLECK, 1967) with 1 *N* $HClO_4$ at 100 °C. The optical density of the hydrolysate was measured at 270 nm in a 1 cm quartz cuvette against 1 *N* $HClO_4$.

1.9. Determination of aliphatic monocarbonic acids

The column chromatographic method of SCHERFIG and co-workers (1968) was used to isolate the carbonic acids from the fermentation broth. The carbonic acids eluted from the columns were converted into hydroxams (ABRAMOVA, 1968). The monocarbonic acid derivatives were separated on a cellulose layer using ethanol–water–acetic acid (70 : 10 : 20) developing solvent. $FeCl_3$ solution was used as detection reagent.

1.10. Methods of bacterium propagation

1.10.1. Propagation in shake cultures in infusion bottles. The method described in detail in an earlier paper (HALÁSZ, 1972) was used.

1.10.2. Propagation in closed fermenter fitted with mixer. Propagation was carried out in the jacketed home-made fermenters of 8000 ml total volume, under periodical gas flow. In order to achieve finer gas distribution gas was introduced under the motor-driven turbine mixer, built in at the bottom of the fermenter (HALÁSZ, 1972).

The turbine mixer served to intensify contact between the gas and the liquid phase. Experience has shown a speed above 1000 rpm not to be expedient, because the intense foaming caused the bacterium cells to separate from the liquid phase by flotation and settle on the wall of the fermenter.

2. Results

2.1. Gas assimilation rate as affected by the increase of CO_2 concentration

The effect of CO_2 concentration in the gas phase on the gas assimilation rate was studied in relation to the pure culture (Strain H_{513}) and the mixed Culture V. The change of pressure in the head-space was controlled at hourly intervals during 24 hours. On the basis of experience gained on methane assimilation, the changes occurring during the 24-hour observation period were classified into three parts: total gas absorption (a) in the first 3 hours, (b) between the 4th and 8th hours and (c) between the 8th and 24th hours. These periods correspond approximately to the lag, exponential and linear phases of growth, resp.

The changes shown on the manometer of the *Warburg* apparatus were converted into gas volume and were corrected for the fluctuations shown by the thermobarometer.

The gas volumes absorbed by Strain H_{513} changed in all the three time periods with changing CO_2 concentrations (Fig. 1).

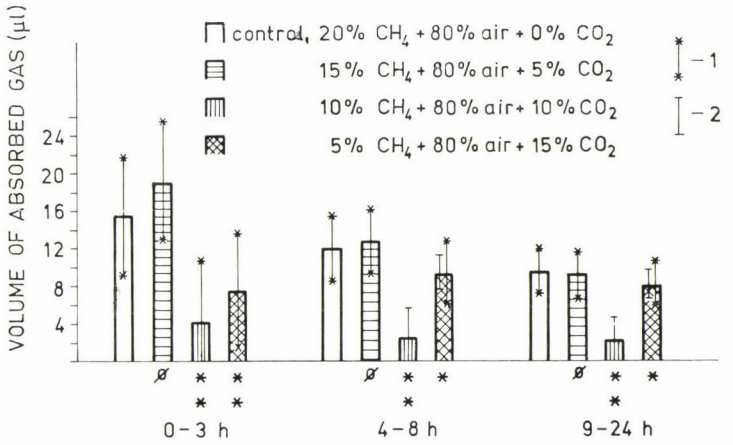

Fig. 1. Gas absorption as affected by CO_2 in the culture of Strain H_{513}. 1: $s_m \cdot t$ = confidence interval at probability level $P_{99\%}$; 2: $s_m \cdot t$ = confidence interval at probability level $P_{95\%}$. Initial viable cell count 10^9 ml^{-1}. Ø = deviation from the control non-significant ($P < 95\%$); * = significantly lower than the control ($P \geq 95\%$);** = highly significantly lower than the control ($P \geq 99\%$)

The values measured in gas mixtures: 80% air + 20% CH_4 and 80% air + 15% CH_4 + 5% CO_2 may not be considered different. In comparison with the control gas absorption was much lower at 10% and 15% CO_2 content. The values obtained at 10% and 15% CO_2 were lower in comparison with those obtained at 5% CO_2.

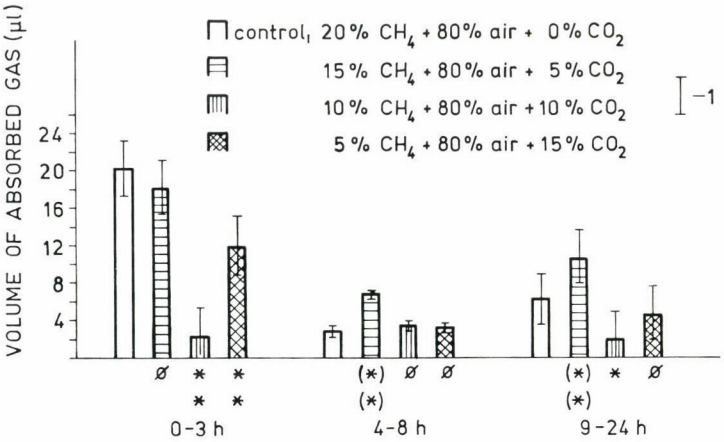

Fig. 2. Gas absorption as affected by CO_2 in Culture V. 1: $s_m \cdot t$ = confidence interval at probability level P 99%. Ø = deviation from the control non-significant ($P \leq 95\%$); * = significantly lower than the control ($P \geq 95\%$); ** = highly significantly lower than the control ($P \geq 99\%$) ** = highly significantly higher than the control ($P \geq 99\%$)

In the first 3 hours of propagation the gas assimilation of Culture V was similar to that shown by Strain H_{513} (Fig. 2).

Gas assimilation under the control conditions was better than in the case of 10% and 15% CO_2 and lower than in the presence of 5% CO_2.

During the 4th–8th-hour period gas assimilation was most favourable in the presence of 5% CO_2. The next favourable was the control, while the lowest assimilation values were obtained in the presence of 10% CO_2.

2.2. The possible utilization of CO_2 as a carbon source

2.2.1. Results obtained with the Warburg apparatus. The effect of CO_2 on methane assimilation was studied in gas mixtures: 5% CO_2 + 15% CH_4 + 80% air; 20% CH_4 + 80% air. The effect of these mixtures on gas assimilation was studied on a synthetic nutrient medium as described in para. 2.1 and in addition to this in nutrient media complemented with two metabolites (methanol and formic acid) of microbiological methane oxidation (Table 1).

The differences in gas assimilation as measured in a gas mixture containing 5% CO_2 and the control, propagated on the synthetic medium and on media complemented with metabolites, resp., were compared. The results were arranged according to the same three time periods as in para. 2.1: 0 to 3rd, 4th to 8th and 8th to 24th hours.

During the first fermentation period 5% CO_2 in the gas atmosphere affected methane assimilation of Strain H_{513} identically in both the metabo-

lite-free and metabolite-containing nutrient media. The same observation was made in the second period (4th to 8th h). In the third period (8th to 24th h), on comparing the differences in gas assimilation, those observed in the presence of methanol were substantially higher than in the metabolite-free and the formic acid-containing nutrients, resp. However, the difference as measured in the metabolite-free medium was higher than that measured in formic acid-containing medium (Table 2).

Table 2

Differences in gas absorption of Strain H_{513} under a gas mixture containing 5% CO_2 and the control gas in metabolite-free and metabolite-complemented nutrient solution

Differences in gas absorption (μl) in different nutrient solutions

Metabolite in the nutrient solution / Time of gas absorption	Control	Methanol	Formic acid	Note
0 to 3rd h	0	0.7157	0	Ø
4th to 8th h	0.2620	0	0.012	Ø
9th to 24th h	0.2145	0.5127	0.0616	*

Ø = difference between columns non-significant (P < 95%)
* = difference between columns significant (P ≥ 95%)

Significance of the differences between gas volumes absorbed in metabolite-free, in methanol-complemented and formic acid-complemented gas atmospheres (9th to 24th h)

n = 22

	Nutrient solution	
	free of metabolite	containing methanol
Methanol-complemented	**	
Formic acid-complemented	*	**

* = difference significant (P ≥ 95%)
** = difference highly significant (P ≥ 99%)

In the case of Culture V the differences in the gas volumes assimilated in the CO_2-containing (5%) and the control gas mixtures, were practically identical in all the three time periods for the media complemented with metabolite, or free of metabolite (Table 3).

Table 3

Differences in gas absorption of Culture V under the gas mixture containing 5% CO_2 and the control in metabolite-free nutrient solutions

Differences in gas absorption (μl) between different nutrient media

Metabolite in the nutrient solution / Time of gas absorption	Control	Methanol	Formic acid	Note
0 to 3rd h	0.5492	0.2383	0.4675	Ø
4th to 8th h	0	0	0.7080	Ø
9th to 24th h	0.0909	0.5928	0.2694	Ø

Ø = difference between columns non-significant ($P < 95\%$)

In the case of Culture S, during the first period of growth (0–3rd h) a difference was observed in the changes in the complemented and in the metabolite-free nutrient media, resp. With this culture the changes occurring upon increasing CO_2 concentration differed in the nutrient solutions complemented with methanol and formic acid, resp., from those observed in the metabolite-free nutrient media. Similar differences were observed in the second (4th to 8th h) and the third (8th to 24th h) period with complemented and non-complemented media (Table 4).

Table 4

Differences in gas absorption of Culture S under the gas mixture containing 5% CO_2 and the control in metabolite-free and metabolite-complemented nutrient solutions, resp.

Differences in gas absorption (μl) in different nutrient solutions

Metabolite in the nutrient solution / Time of gas absorption	Control	Methanol	Formic acid	Note
0 to 3rd h	0.41	3.0667	2.5583	*
4th to 8th h	0.1168	2.2396	0.8082	*
9th to 24th h	0.1790	2.2225	2.0408	*

* = difference between columns significant ($P \geq 95\%$)

Significance of differences between gas volumes recorded under different gas atmospheres in metabolite-free, in methanol- and formic acid-containing nutrient solutions, resp. (0 to 3rd h)

	Nutrient solution free of metabolite	Methanol in the nutrient solution
Methanol-complemented	**	
Formic acid-complemented	**	*

(4th to 8th h)

	Nutrient solution	
	without metabolite	with metabolite
Methanol-complemented	*	
Formic acid-complemented	Ø	Ø

(9th to 24th h)

	Nutrient solution	
	free of metabolite	containing metabolite
Methanol complemented	*	
Formic acid complemented	*	Ø

2.2.2. Results obtained in shake cultures. The effect of the composition of the gas mixture on cell growth in media complemented with methanol or formic acid, resp., was studied in shake cultures. The following gas mixtures were compared:

$$0\%\ CH_4 - 0\%\ CO_2 - 100\%\ \text{air}$$
$$20\%\ CH_4 - 0\%\ CO_2 - 80\%\ \text{air (control)}$$
$$15\%\ CH_4 - 5\%\ CO_2 - 80\%\ \text{air}$$
$$10\%\ CH_4 - 10\%\ CO_2 - 80\%\ \text{air}$$

In the case of Strain H_{513} in methanol-containing nutrient solution the gas mixture of 5% CO_2 content proved the most favourable. In a gas atmosphere of 100% air conditions of growth were more favourable than under conditions of the control, while a gas atmosphere containing 5% CO_2 exerted approximately the same effect. The difference between the effect of the control and the mixture of 10% CO_2 concentration was insignificant (Table 5).

Table 5

Cell growth of Strain H_{513} as affected by CO_2 in methanol-containing nutrient media

Cell growth ($\Delta E \cdot 100$)

Composition of the gas mixture	100% air	Control	5% CO_2	10% CO_2	Note
$\Delta E \cdot 100$	28.833	16.0	27.333	19.667	*

* = difference between columns significant ($P \geq 95\%$)

Significance of differences between cell growth data under various gas atmospheres

	Air	Control	5% CO_2
Control	*		
5% CO_2	Ø	*	
10% CO_2	*	Ø	*

Ø = difference non-significant
* = difference between columns significant ($P \geq 95\%$)

There was no significant difference in the growth of Strain H_{513} in formic acid-complemented nutrient solution under all the gas mixtures studied (Table 6).

Table 6

Effect of CO_2 upon the cell growth of Strain H_{513} in formic acid-complemented nutrient medium
Cell growth ($\Delta E \cdot 100$)

Composition of the gas mixture	100% air	Control	5% CO_2	10% CO_2	Note
$\Delta E \cdot 100$	0.3333	1.1667	1.0	1.6667	Ø

Ø = difference between columns non-significant ($P < 95\%$)

Culture V grew in nutrient solutions containing methanol or formic acid at about the same rate under all the gas mixtures investigated (Tables 7 and 8).

Table 7

Effect of CO_2 upon the cell growth of Culture V in methanol-complemented nutrient medium
Cell growth ($\Delta E \cdot 100$)

Composition of the gas mixture	100% air	Control	5% CO_2	10% CO_2	Note
$\Delta E \cdot 100$	16.750	8.8750	17.750	17.125	Ø

Ø = difference between columns non-significant ($P < 95\%$)

Table 8

Effect of CO_2 upon the cell growth of Culture V in formic acid-complemented nutrient medium
Cell growth ($\Delta E \cdot 100$)

Composition of the gas mixture	100% air	Control	5% CO_2	10% CO_2	Note
$\Delta E \cdot 100$	1.3333	2.3333	1.6667	1.3333	Ø

Ø = difference between columns non-significant ($P < 95\%$)

Culture S proliferated at various rates in methanol-complemented nutrient medium under the different gas mixtures. Conditions for growth were most favourable in a 100% air atmosphere. The difference in growth between a gas atmosphere of 100% air and a gas mixture containing 10% CO_2 was not significant.

Except for the culture under 100% air, the control did not differ from the fermentations under other gas mixtures. Difference was not observed in the effect of the gas mixtures containing 5% or 10% CO_2 (Table 9).

Table 9

Effect of CO_2 upon cell growth of Culture S in methanol-complemented nutrient medium
Cell growth (Δ E · 100)

Composition of the gas mixture	100% air	20% CH_4–80% air	5% CO_2	10% CO_2	Note
Δ E · 100	17.500	9.2500	8.2500	12.750	*

* = difference between columns significant ($P \geq 95\%$)

Significance of differences between cell growth data under various gas atmospheres

	100% air	Control	5% CO_2
Control	*		
5% CO_2	*	Ø	
10% CO_2	Ø	Ø	Ø

Ø = difference non-significant
* = difference significant ($P \geq 95\%$)

In the propagation of Culture S in nutrient solutions with added formic acid there was no difference in growth under the different gas mixtures (Table 10).

Table 10

Effect of CO_2 upon the cell growth of Culture S in formic acid-complemented nutrient medium
Cell growth (Δ E · 100)

Composition of the gas mixture	100% air	Control	5% CO_2	10% CO_2	Note
Δ E · 100	1.0000	0.7500	2.0000	1.5000	Ø

Ø = difference between columns non-significant ($P < 95\%$)

2.3. Main characteristics of fermentation as affected by CO_2 concentration

2.3.1. Growth as affected by CO_2 concentration. The effect of CO_2 concentration on cell growth was characterized by the change in cell density in the first hour of propagation (for the lag phase of growth) and by the change in cell density at the 8th hour of fermentation (for the exponential phase).

The results obtained with the three cultures were classified as follows.

Cell growth in the first hour of fermentation was satisfactory if cell growth exceeded 20% of the initial concentration. Between 10% and 20% it was considered medium and below 10% low.

According to the classification as described above there was no difference observed under gas mixtures containing 20% CH_4 + 80% air and 15% CH_4 + 5% CO_2 + 80% air, resp., in the initial phase of growth (Tables 11, 12 and 13).

Table 11

Effect of CO_2 on the lag phase of growth of Strain H_{513} propagated in the fermenter
Table m × n

Growth / Gas phase	Satisfactory above 20%	Medium 20–10%	Low below 10%	Sum
Control	3	1	2	$n_1 = 6$
5% CO_2	3	3	0	$n_2 = 6$
sum of frequency	$m_1 = 6$	$m_2 = 4$	$m_3 = 2$	$N = 12$ = total

$\chi^2 = 3.0$ $\chi^2_{95} = 5.991$ Difference between the effects of the two gas phases non-significant ($P < 95\%$)

Table 12

Effect of CO_2 on the lag phase of growth of Culture V propagated in the fermenter
Table m × n

Growth / Gas phase	Satisfactory above 20%	Medium 10–20%	Low below 10%	Sum
Control	5	0	1	$n_1 = 6$
5% CO_2	4	1	1	$n_2 = 6$
sum of frequency	$m_1 = 9$	$m_2 = 1$	$m_3 = 2$	$N = 12$ = total

$\chi^2 = 3.1068$ $\chi^2_{95} = 5.991$ Difference between the effects of the two gas phases non-significant ($P < 95\%$)

Table 13

Effect of CO_2 on the lag phase of growth of Culture S propagated in fermenter
Table m×n

Growth / Gas phase	Satisfactory above 20%	Medium 10–20%	Low below 10%	Sum
20% CH_4 + 80% air	2	0	1	$n_1 = 3$
5% CO_2 + 15% CH_4 + + 80% air	2	0	1	$n_2 = 3$
sum of frequency	$m_1 = 4$	$m_2 = 0$	$m_3 = 2$	N = 12 = total

$\chi^2 = 0$ The difference between the two gas phases is non-significant (P < 95%)

In the exponential phase the following classification was applied.

Growth was considered satisfactory when optical density was above 50% of the initial value. It was considered medium between 30 and 50% of the initial value and low under 30%.

Applying the above classification no difference was found between growth under the two gas mixtures (Tables 14, 15 and 16).

On comparing the effect of gas mixtures of 5 and 10% CO_2 concentration, in the lag phase the difference found was insignificant, while in the expo-

Table 14

Effect of CO_2 on cell density of Strain H_{513} in 8-h fermentations in the fermenter
Table m×n

Cell density / Gas phase	Satisfactory above 20%	Medium 10–20%	Low below 10%	Sum
Control	0	3	3	$n_1 = 6$
5% CO_2	0	3	3	$n_2 = 6$
sum of frequency	$m_1 = 0$	$m_2 = 6$	$m_3 = 6$	N = 12 = total

$\chi^2 = 0$ There is no difference between the effect of the two gas phases (P < 95%)

Table 15

Effect of CO_2 on cell density of Culture V in 8-h fermentations in the fermenter
Table m×n

Cell density / Gas phase	Satisfactory above 20%	Medium 10–20%	Low below 10%	Sum
Control	3	3	1	$n_1 = 7$
5% CO_2	3	4	0	$n_2 = 7$
sum of frequency	$m_1 = 6$	$m_2 = 7$	$m_3 = 1$	N = 14 = total

$\chi^2 = 1\ 1424$ $\chi^2_{95} = 5.991$ Differences between gas phases are non-significant (P < 95%)

Table 16

Effect of CO_2 on cell density of Culture S in 8-h fermentation in the fermenter
Table m×n

Cell density / Gas phase	Satisfactory above 20%	Medium 10–20%	Low below 10%	Sum
Control	0	1	1	$n_1 = 2$
5% CO_2	1	0	1	$n_2 = 2$
sum of frequency	$m_1 = 1$	$m_2 = 1$	$m_3 = 2$	$N = 4$ = total

$\chi^2 = 2.0$ $\chi^2_{95} = 5.99$ Differences are non-significant ($P < 95\%$)

Table 17

Effect of gas compositions with 5% and 10% CO_2, resp., on growth of Culture S propagated in the fermenter
Lag phase Table m×n

Cell growth / Gas phase	Satisfactory above 20%	Medium 10–20%	Low below 10%	Sum
5% CO_2	1	0	1	$n_1 = 2$
10% CO_2	0	0	1	$n_2 = 1$
sum of frequency	$m_1 = 1$	$m_2 = 0$	$m_3 = 2$	$N = 3$ = total

$\chi^2 = 1.332$ $\chi^2_{95} = 3.991$ Differences are non-significant ($P < 95\%$)

nential phase differences were substantial and 5% CO_2 could be considered definitively more advantageous (Table 17).

2.3.2. The effect of CO_2 concentration on the quality and quantity of acid metabolites formed during methane assimilation. As seen in Table 18, under gas mixtures containing CO_2 both formic acid and acetic acid concentrations are higher than in fermentations without CO_2. The difference between concentrations is significant.

In all the samples taken in the 30th, 60th, 120th and 240th minutes of fermentation the formic and acetic acid concentrations were higher under the gas mixture with 5% CO_2 content.

2.3.3. The effect of CO_2 concentration upon the main components of the biomass formed during methane assimilation. The protein content of the biomass under a gas mixture of 5% CO_2 + 15% CH_4 + 80% air was higher than in the control with both Strain H_{513} and Culture V. The protein content related to the solids content of Culture S did not change as a function of CO_2 (Table 19).

The nucleic acid concentration in the biomass varied with the different cultures as a function of the CO_2 content of the gas mixture (Table 20).

Table 18

Formic acid and acetic acid concentrations during fermentation as affected by gas compositions: 20% CH_4 + 80% air and 5% CO_2 + 15% CH_4 + 80% air resp.

Time of sampling, min	Culture V				Strain H_{513}				Culture S			
	Gas composition											
	20% CH_4 80% air		5% CO_2 15% CH_4 80% air		20% CH_4 80% air		5% CO_2 15% CH_4 80% air		20% CH_4 80% air		5% CO_2 15% CH_4 80% air	
	Acid concentration in the fermentation broth mg/100 ml											
	formic acid	acetic acid	formic acid	acetic acid	formic acid	acetic acid	formic acid	acetic acid	formic acid	acetic acid	formic acid	acetic acid
30	9.20	10.75	21.88*	27.28*	2.76	3.60	3.68	4.70	5.79	9.60	7.03*	9.96*
60	7.64	12.40	14.90	19.84	2.48	3.24	3.22*	4.20*	5.58	4.68	5.70*	7.44*
120	5.34	10.56	10.94*	13.80*	2.39	3.12	2.94	3.84	1.10	1.44	2.02*	2.64*
240	4.13	6.96	8.46*	10.56*	1.47	1.96	1.75	2.28	1.01	1.32	1.56	2.52*

Fermentation values are the averages of five fermentations, each
* = difference significant at the level of P = 95 %

Table 19

Protein content of the biomass when propagated under a gas mixture containing 5% CO_2 and the control, resp.

	Strain H_{513}		Culture V		Culture S	
	Control	5% CO_2	Control	5% CO_2	Control	5% CO_2
Protein content as percentage of cell solids	50.0	53.1	51.6	55.0	49.6	47.0
	58.1	58.1	58.0	60.5	39.0	40.5
	51.3	54.2	58.0	62.0	—	—

The protein content of the biomass grown under the gas atmosphere of 5% CO_2 concentration is significantly higher ($P \geq 95\%$) than that grown under the control

Table 20

Nucleic acid (RNA) content of the biomass grown under a gas atmosphere of 5% CO_2 and under the control, resp.

	Strain H_{513}		Culture V		Culture S	
	Control	5% CO_2	Control	5% CO_2	Control	5% CO_2
Nucleic acid as % of cell solids	13.2	15.3	7.8	9.0	11.4	7.9
	13.0	14.2	7.9	9.2	13.8	7.5
			10.2		9.0	
			8.9		9.0	

On the basis of the *t* test the differences between pairs of data are not considered significant ($P < 95\%$); there are no sufficient data available to evaluate results in relation to each strain

The nucleic acid concentration in the biomass of Strain H_{513} was higher at 5% CO_2 concentration, in the case of Culture V slightly higher, than under control conditions, while with Culture S the RNA concentration was higher in the control fermentation.

3. Conclusions

The effect of CO_2 concentration in the gas mixture was different or partly different with the three methane-assimilating cultures. Therefore conclusions are summarized in relation to individual cultures.

3.1. Effect of the CO_2 concentration of the gas mixture on Strain H_{513}

Of the gas mixtures investigated the one containing 5% CO_2 exerted the same effect as the control mixture, while the other mixtures had a significantly less favourable effect, as was shown in both the experiments, in the *Warburg* respirometer and in shake cultures.

The results of the fermenter experiments are in agreement with those on rate of gas absorption. Differences are not significant either in the lag phase or in the exponential phase between growth under a gas mixture containing 5% CO_2 and under the control.

However, higher CO_2 concentrations affect methane utilization as shown by the increase in metabolite concentration and in the protein content of the biomass. Both the metabolite concentration in the fermentation broth and the protein concentration in the biomass are significantly higher under the gas mixture of 5% CO_2 than in the control. As regards nucleic acid concentration in the biomass, available data are insufficient to evaluate the differences in relation to the cultures.

When metabolite was added to the nutrient medium the difference in the values measured in the CO_2-containing and in the control gas mixtures between the 9th and 24th hour of respiration the methanol-containing nutrient proved to be significantly more favourable.

Observations made during gas absorption show that in the presence of CO_2 methane assimilation is at least stimulated. In a gas mixture containing 5% CO_2, methane concentration is 25% lower than in the control, therefore, a lower growth rate and gas absorption would be expected.

However, cell growth experiments permit of the conclusion that gas absorption is accelerated, too. The approximately identical metabolite concentration in a gas mixture of lower (by 25%) methane content indicates a substantially stimulated process, too.

Correlating these observations with those obtained in the methanol-containing nutrient solution the conclusion may be drawn that the energy

requirement of CO_2 incorporation is provided by methane oxidation and this occurs in the intermediate phase of methanol assimilation. By increasing the CO_2 concentration above 5% at the cost of the methane concentration, the latter is reduced to half or even to a quarter of the original value and thus the rate of gas absorption and the source of energy of CO_2 incorporation decreases. The approximately identical level of acid metabolites confirms that methane oxidation provides energy for CO_2 incorporation. The increase in protein concentration may be explained by the incorporation of CO_2 through condensation reactions.

3.2. Effect of CO_2 concentration of the gas mixture on Culture V

In metabolite-free fermentation, gas assimilation by Culture V unambiguously increases the rate of methane oxidation up to a concentration of 5% CO_2. Similar results were obtained in the cell growth experiments.

Increase in the protein concentration of the biomass obtained by fermentation (by about 8–10% of that of the control), doubling of the acid metabolites in the initial phase (30th and 60th min), then their increase to above 50% all indicate that the presence of CO_2 exerts a substantial stimulating effect on the metabolism of the culture.

This phenomenon may be explained as in the case of Strain H_{513}, by the oxidation of CH_4 providing the energy requirement of CO_2 incorporation. The identical gas absorption rates and cell growth, as well as the disproportionate increase of acid metabolites in relation to the other cultures indicate that methane oxidation by Culture V goes ahead with substantially lower energy liberation.

3.3. Effect of the CO_2 concentration of the gas mixture on Culture S

During the whole of the fermentation process of Culture S, in both the methanol- and the formic acid-containing nutrient solutions, significant changes are brought about by the presence of 5% CO_2 as compared to the metabolite-free nutrient.

Cell density in the methanol-containing nutrient solution was significantly higher under 100% air than under gas mixtures of 5% CO_2 + 15% CH_4 + 80% air and of 20% CH_4 + 80% air, resp., when fermented in shake culture. The effects of the control and of the gas mixture containing 5% CO_2 are not considered different. The difference between the effects of 100% air and of 10% CO_2 was not significant either.

When propagated in the fermenter (in metabolite-free medium) the gas phase containing 10% CO_2 exerted a significantly adverse effect as compared to the one with 5% CO_2. The gas atmosphere of 5% CO_2 + 15% CH_4 +

80% air did not induce a significantly higher protein content in the biomass. while causing a significant increase in the concentration of acid metabolites.

On the basis of the above, methanol seems to be the most probable energy source of CO_2 incorporation. CH_4 could also be considered a source of the necessary energy, however, the acceptors needed to the incorporation of CO_2 come from the metabolism of methanol, thus CO_2 incorporation is highly enhanced by the addition of methanol.

On comparing cell densities obtained with 100% air or with 10% CO_2 + 10% CH_4 + 80% air, these may be considered identical, while comparing the results in 100% air and in 5% CO_2, resp., the latter resulted in lower cell densities. Thus incorporation of CO_2 with Culture S seems to be a slower process than with Culture V or Strain H_{513} (higher requirement for added CO_2).

Experiments carried out with the three cultures have shown that the increase of the CO_2 concentration does not affect the lag phase of growth. In comparison with the control CO_2 concentrations of 10% and 15% affected methane assimilation unfavourably. These observations differ from the findings of SMIRNOVA (1971) related to the lag phase and from those of SHEEHAN and JOHNSON (1971) who found that CO_2 concentrations up to 55% do not harm fermentation.

The observation that the three cultures reacted differently to the change in CO_2 concentration support the findings of VAN DER LINDEN and THIJSEE (1965), who noted that the CO_2 binding capacity of different strains varied.

The incorporation of CO_2 requires an energy source and acceptors which may come from methane or methanol. It was shown in this study that the energy production from methane varied with the various cultures.

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CORROSION OF STEEL AND ALUMINIUM CONSTRUCTION MATERIALS IN DIFFERENT FOOD MEDIA

PART I. — ALCOHOL-FREE FOODS

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Weight losses of metals to be used as construction materials in the manufacture of equipment for the food industries is given in the manuals in g m^{-2} units. However, the time period of the experiments in which these data were obtained are not disclosed. The experiments were carried out either with a view to changes in the metallic construction material in question or changes in the food medium in contact with the metal.

In the experiments herein described an attempt was made to reconcile the two aspects. Carbon steel and aluminium were used as model metals and their corrosion with time in alcohol-free, liquid and semi-liquid foods and model solutions was investigated. In the experiments the liquid phase was stationary. At the same time the iron and aluminium contents of the food media and model solutions, used as corrosive medium was determined. An attempt was made to establish a correlation between the metal content of the given food medium on one hand, and the weight loss of the test specimens on the other.

Rate of carbon steel corrosion was found to be higher in the initial phase, slowing down after 30 days to become finally stabilized. Thus data obtained in experiments extending beyond 30 days give more reliable help in constructing storage tanks.

The corrosion mechanism of aluminium differs from that of carbon steel completely, particularly in media containing halogen ions, too. In this case pitting, spot corrosion and the formation of grooves have to be taken into account.

The weight loss of the specimens and the metal content in the aggressive medium did not show direct correlation, particularly in the case of aluminium. This is probably due to the lower precision of aluminium determination.

Studies of the corrosive effect of food media upon metals have proven that the damage arising from the contamination of foods may be in many cases of greater importance than the direct damage caused by the corrosion of the metals. SELBY (1955) found that the presence of non-toxic metals, widely used in the food industries, may lend a metallic taste to certain foods. Apart from this they can cause or catalyze changes (change of colour, rancidity) detrimental to food quality.

In view of the afore said to select the appropriate construction material for equipment designed for use in the food industries a knowledge of the corrosive effect of the various food materials upon the metals is essential. Data in the manuals on the weight changes of metals are usually given in g m^{-2} day^{-1} or g m^{-2} h^{-1} values. However, the time period of the experiments in which these data were obtained is usually not disclosed (RITTER, 1958).

In his paper on this subject MIKASINOVIČ (1959) describes the corrosive effect of foods upon a variety of metals but states only in relation to the data on the corrosion of nickel that they were the result of a 30-day experiment.

In her book, AVDIEVA (1962) describes a number of studies on the corrosion of metals in food media, however, the duration of these experiments is disclosed only in a few cases. Only a single case is mentioned where the experimental period extended over 500 hours.

Hungarian authors studied mainly the behaviour of aluminium under the conditions of the food industry and consider it completely or practically resistant to corrosion (BÁCSKAI, 1949; DOMONY, 1958; MONORI, 1958; GYÖNÖS, 1960; LICHTENBERGER, 1961). Results of these experiments were obtained in experiments lasting a few hours or days, at most 30 days. The attention of the authors extended only over the aluminium samples the corrosive effect of which was investigated and did not cover the food medium.

An interesting analytical test series was conducted by KOCH and BREKER (1958). The effect of the corrosion of aluminium cans on apple juice and grape juice preserved by sulphur dioxide was studied. Changes of the juices and the metal ion content in the aggressive medium were examined as a function of time. The weight change of the cans was not checked directly.

In the first experiments aluminium cans of 400 ml volume were used. A part of the cans containing the juice was kept at 37 °C for 8 and 31 days, resp., the rest was stored at 20 °C for 1, 2, 3 or 4 months, resp.

At the end of the experimental period the apple juice was found to have lost its aroma, while the grape juice was markedly repugnant and unfit for consumption. In the samples stored at 20 °C the change occurred during a month, while in those kept at 37 °C in 8 days.

The apple juice had after a storage period of 31 days a metallic off-flavour and the cans containing grape juice underwent deformation by hydrogen development. Beside hydrogen, hydrogen sulphide was also observed in the cans.

An aluminium content of 40 mg l^{-1} was found after 8 days and 260 mg l^{-1} after 31 days in the apple juice.

On the basis of these preliminary experiments KOCH and BREKER (1958) drew conclusions as to the corrosion of aluminium containers used for the storage of juices from the aluminium concentration in the juice. In the knowledge of the dimensions of the container and the aluminium concentration in the juice they calculated the corrosion of the aluminium container in unit time with the help of an equation.

KOCH and BREKER (1958) tried to find a correlation between the acid content of the juice and the degree of corrosion of the container. However, the number of results in favour of the correlation was about the same as against it.

The authors mentioned above examined either the qualitative and quantitative changes of the metallic construction material or the change in the food medium contacting the metal, only.

In the experiments here described we tried to combine the two aspects. The corrosion with time of carbon steel and aluminium was studied and an assay was made to establish the shortest experimental period permitting of drawing conclusions as to the corrosion of the given construction material in the food medium in question. (In testing carbon steel we did naturally not forget the fact that, in practice, carbon steel can not get in direct contact with food. However, since it seemed a very satisfactory model metal, it was used in the preliminary experiments.) The correlation between the weight change of the test specimens and the metal concentration in the food used as aggressive medium was also investigated.

1. Materials and methods

1.1. Materials

The test specimens used in the experiment were made of S3F carbon steel plate (HUNGARIAN STANDARD, 1971) and of Al 99.5 foundry aluminium. The nominal thickness of the plates was 2 mm and the size of the test specimens 30×50 and 50×100 mm.

The following model solutions and foods were used as corrosive media:

a) Diluted brine
30 g l^{-1} NaCl
20 g l^{-1} acetic acid
1 g l^{-1} sodium benzoate
1.5 g l^{-1} alum

b) Brine of high common salt content
200 g NaCl
1.5 g sodium benzoate
4.4 g citric acid
0.5 g ascorbic acid
762 ml water
31.2 ml 96% acetic acid (KARDOS & SZENES, 1972)

c) Meat pickle (LŐRINCZ *et al.*, 1961)
323.4 g NaCl
6.6 g KNO_3
1000 ml water

d) Sour-cherry juice (Product of BUDAPEST CANNING FACTORY)
Preserved with sorbic acid

e) Starch syrup (BUDAPEST CHOCOLATE FACTORY)

f) Sunflower seed oil freshly refined (RÁKOSPALOTA VEGETABLE OIL FACTORY) (HUNGARIAN STANDARD, 1972)
g) Lard (commercial) (HUNGARIAN STANDARD, 1973a)
h) Vegetable oil (Margarine) (HUNGARIAN STANDARD, 1962c)
i) Pasteurized milk (commercial, packed in plastic pouches) (HUNGARIAN STANDARD, 1973b)

1.2. Methods

1.2.1. Preparation of the test specimens. The surface of carbon steel test specimens was polished and defatted with trichloro-ethylene.

The surface of the aluminium test specimens was lightly polished and defatted with a 3% solution of 60 °C of Alupon T.

1.2.2. Corrosion tests. Five test specimens of 30×50 mm size, made of the same material were placed in a container filled with the corrosive food medium and kept under conditions resembling storage of the food (in stationary fluid at room temperature or under cooling). To study the time-course of corrosion the five specimens were removed at predetermined points of time, inspected and their weight loss determined. The metal concentration in the aggressive medium was simultaneously determined.

When milk was used as corrosive medium it was replaced every 72 or 96 hours. Thus this test may be considered a cycle test, because the test specimens were washed in hot "Csepel 12" solution and immersed in 0.2% "Chloramin T" disinfectant for 10 min at the beginning of each cycle.

In a parallel experiment the milk was clotted at room temperature and subsequently the test specimens were kept in the sour milk in a single cycle for 7 days in a cold place.

The test specimens were checked after 7, 14, 21, 30, 90 and 180 days, in certain cases after 1 and 3 days.

1.2.3. Determination of the iron or aluminium concentration in the food medium. The iron or aluminium concentration in the food serving as the aggressive medium was determined by the methods given in HUNGARIAN STANDARD (1962a) and HUNGARIAN STANDARD (1962b), after wet-ashing with nitric acid and perchloric acid (SPANYÁR & KEVEI, 1961).

For the sake of control the iron concentration in sour-cherry juice was determined also by atom absorption spectrophotometry in a *Perkin-Elmer* 290/B type apparatus.

2. Results

Although all the food media listed in para. 1.1 were applied in corrosion tests, evaluable results were obtained only in the following: sour-cherry juice, brine of high salt content, diluted brine, meat pickle, pasteurized milk and

clotted milk. In the other tests the weight changes under the given conditions were within the systematic error, therefore could not be evaluated.

Weight loss of the carbon steel test specimens in sour-cherry juice, brine of high salt content, and in meat pickle as a function of time is shown in

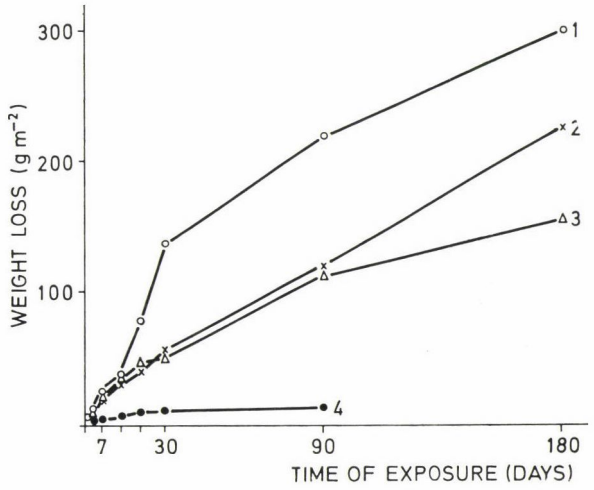

Fig. 1. Weight loss of carbon steel test specimens in foods and model solutions. 1. Sour-cherry juice; 2. Brine of high salt concentration; 3. Diluted brine; 4. Meat pickle

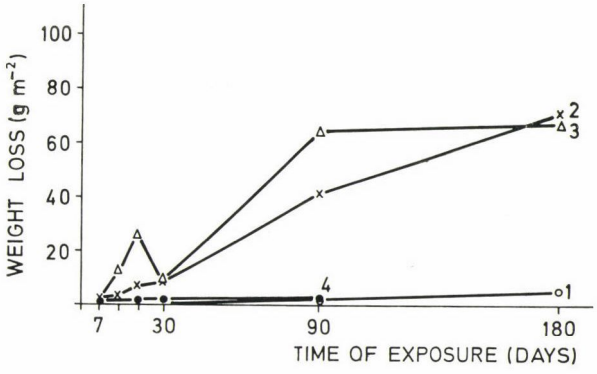

Fig. 2. Weight loss of aluminium test specimens in foods and model solutions. 1. Sour-cherry juice; 2. Brine of high salt concentration; 3. Diluted brine; 4. Meat pickle

Fig. 1. Weight loss of the aluminium specimens in the same liquids is shown in Fig. 2.

Along the edges of the aluminium test specimens, kept in the brine of high salt content for 180 days, deep pits could be observed. Inside these pits, in rolling direction, a laminar structure was visible.

On the surface of the aluminium test specimens kept in the diluted medium for 180 days longitudinal grooves were formed, also in rolling direction. The test specimens are shown in Fig. 3.

The iron concentration in the foods and model solutions used as corrosive media is shown in Fig. 4, as a function of time, while the effect of time on the aluminium concentration in Fig. 5.

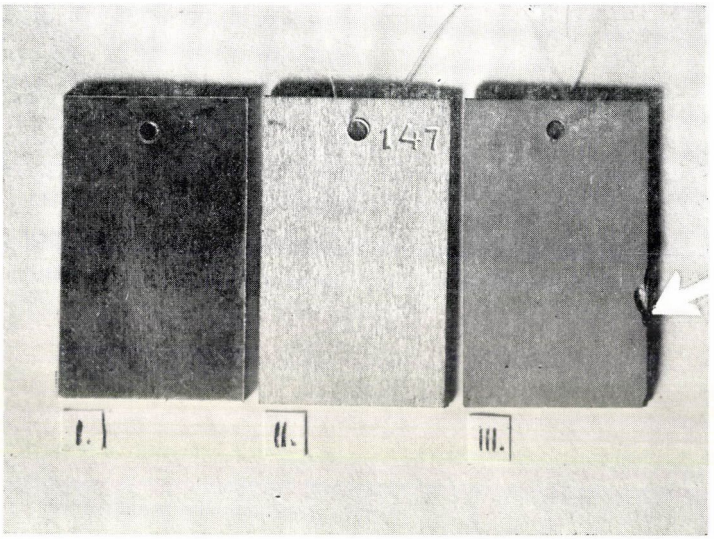

Fig. 3. Aluminium test specimens kept in brine for 180 days. I — control; II — specimen kept in diluted brine; III — specimen kept in brine of high salt concentration. In the place indicated by the arrow a deep groove was formed

The weight loss of the carbon steel and aluminium test specimens in milk and clotted milk is illustrated in Fig. 6 separately, because in these experiments the milk was replaced regularly and also because the experimental periods were shorter.

The results of measurements were used to calculate the weight loss of test specimens per day within the given experimental period and the corrosion rate for both carbon steel and aluminium was given in g m^{-2} day^{-1}. In manuals this unit is generally used to characterize the corrosion resistance of a construction material.

Unit values belonging to the different experimental periods are shown in Figs. 7, 8 and 9.

It can be seen in Fig. 7 that the corrosion rate of the carbon steel test specimens is not a constant value but, gradually decreasing from a high initial value, approaches a limit.

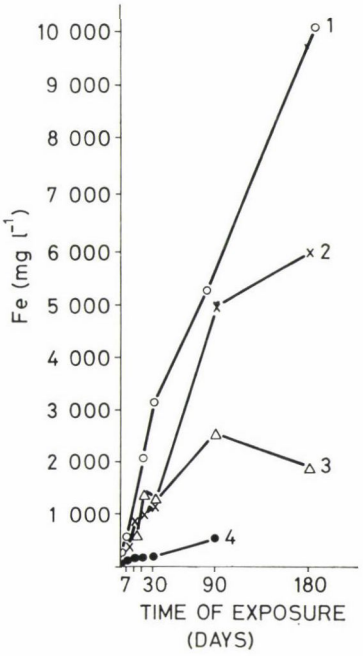

Fig. 4. Iron concentration in food materials and in model solutions as a function of the test period. 1. Sour-cherry juice; 2. Brine of high salt concentration; 3. Diluted brine; 4. Meat pickle

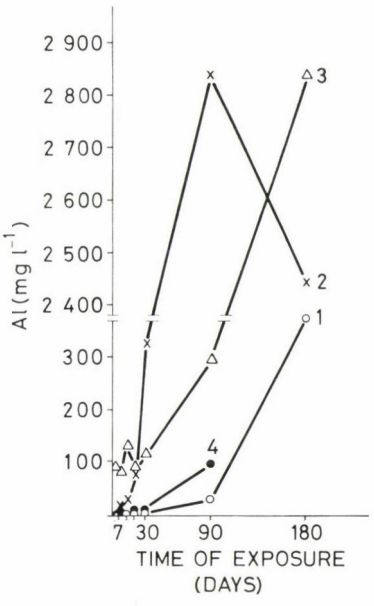

Fig. 5. Aluminium concentration in food media and in model solutions as a function of time. 1. Sour-cherry juice; 2. Brine of high salt concentration; 3. Diluted brine: 4. Meat pickle

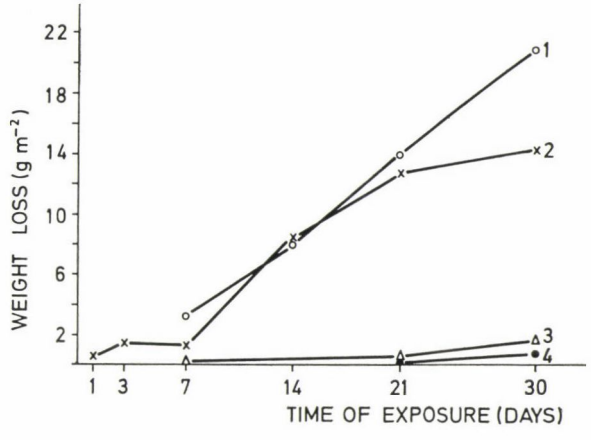

Fig. 6. Weight loss of carbon steel and aluminium test specimens in milk and curdled milk as a function of time. 1. Fe in curdled milk; 2. Fe in milk; 3. Al in milk; 4. Al in curdled milk

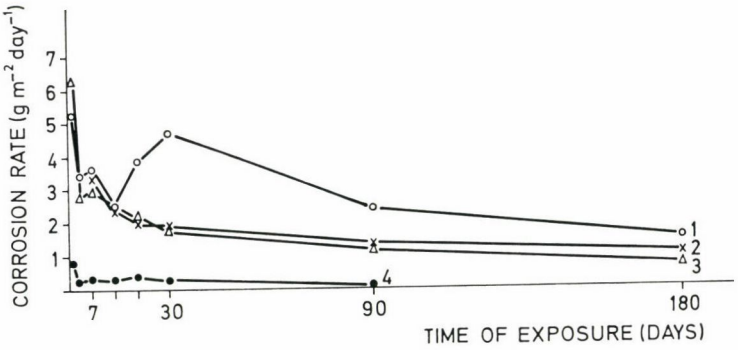

Fig. 7. Corrosion rate of carbon steel specimens as calculated from their weight loss, as a function of time. 1. Sour-cherry juice; 2. Brine of high salt content; 3. Diluted brine; 4. Meat pickle

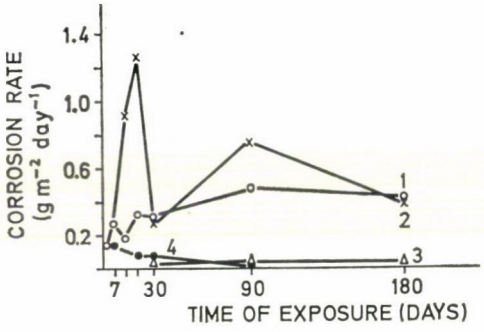

Fig. 8. Corrosion rate of aluminium specimens as calculated from their weight loss, as a function of time. 1. Sour-cherry juice; 2. Brine of high salt content; 3. Diluted brine; 4. Meat pickle

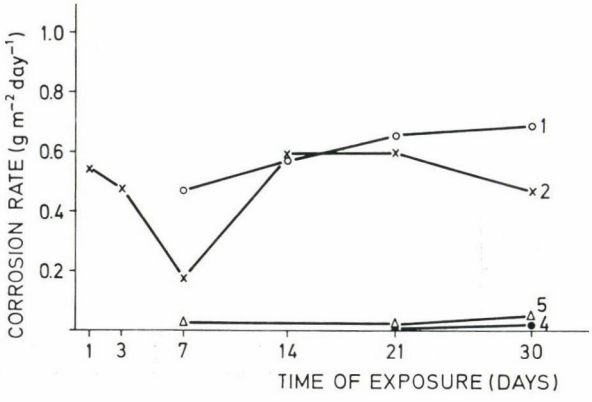

Fig. 9. Corrosion rate of carbon steel and aluminium test specimens as calculated from their weight loss in milk and curdled milk as a function of time. 1. Fe in curdled milk; 2. Fe in commercial milk; 3. Al in commercial milk; 4. Al in curdled milk

The same data for aluminium when plotted (Fig. 8) did not by any means show such a uniform character. Weight changes of the aluminium test specimens in the two brines are alternately increasing and decreasing. This seems to prove the fact that, in a halogen ion-containing medium, the corrosion of aluminium follows a different pattern from that in a medium free of halogen-ion.

The iron and aluminium concentration in the foods and model solutions used as aggressive media was determined. These concentration data were compared to the weight loss as measured in the test specimens. Data are shown in Figs. 10 to 13.

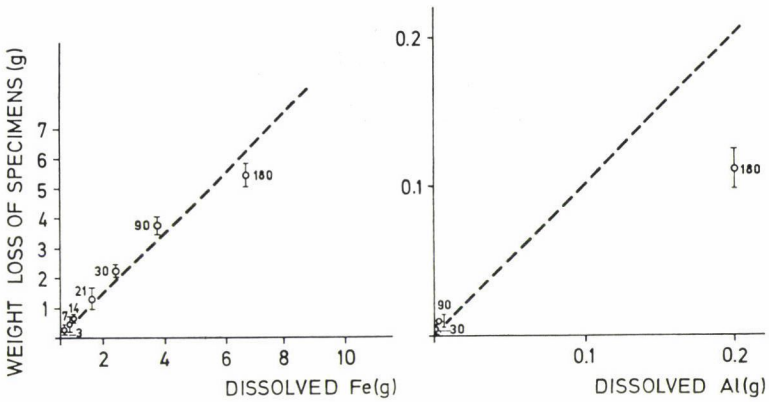

Fig. 10. Comparison of the weight loss of (5) carbon steel or (5) aluminium specimens in sour-cherry juice and of the total amount of iron or aluminium dissolved in the juice, as a function of time. The dashed line represents the correlation to be expected with the weight loss of metal (Fe or Al) specimens, the numbers indicatethe time of exposure (days) and the vertical bars the standard deviation

The correlation between the weight loss of carbon steel specimens and the iron content of the medium is more unambiguous than the correlation of aluminium specimens and the aluminium content of media. This is explained by the different mechanism of aluminium corrosion and by a greater element of uncertainty in aluminium determination methods.

To check the results, the experiments were repeated with test specimens of larger surface in sour-cherry juice. The experimental conditions were identical, only the sour-cherry juice was of another year. Weight loss of the carbon steel and aluminium specimens as a function of time is shown in Fig. 14.

On comparing the curves of Fig. 14 and those in Figs. 1 and 2 related to sour-cherry juice, the agreement is found satisfactory.

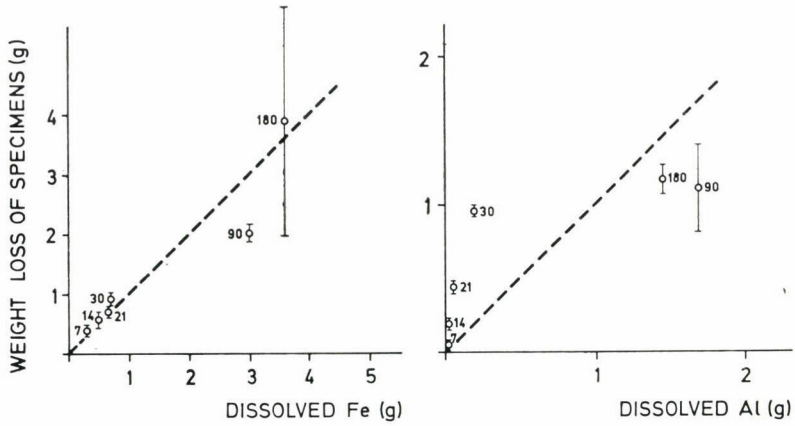

Fig. 11. Comparison of the weight loss of (5) carbon steel or (5) aluminium specimens exposed to brine of high salt content and of the total amount of iron or aluminium dissolved in the brine as a function of time. The dashed line represents the correlation to be expected with the weight loss of metal (Fe or Al) specimens, the numbers show the time of exposure (days) and the vertical bars the standard deviation

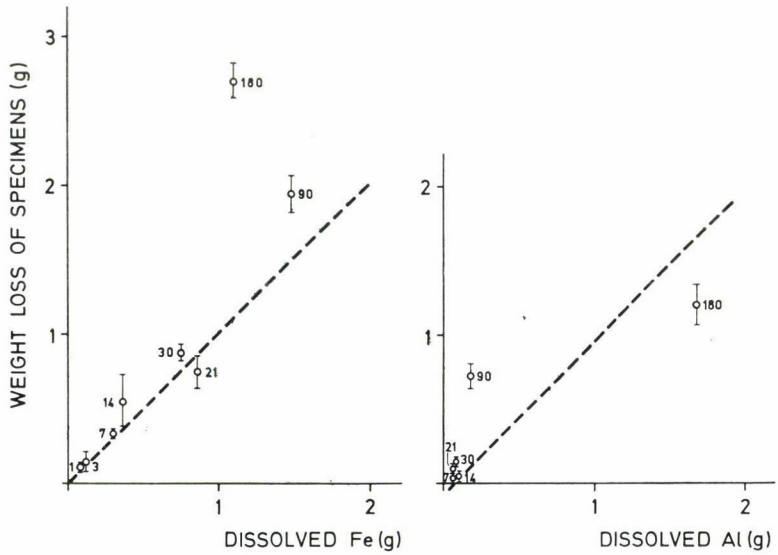

Fig. 12. Comparison of the weight loss of (5) carbon steel or (5) aluminium specimens exposed to diluted brine and of the total amount of iron or aluminium dissolved in the brine, as a function of time. The dashed line represents the correlation to be expected with the weight loss of metal (Fe or Al) specimens, the numbers show the time of exposure (days) and the vertical bars the standard deviation

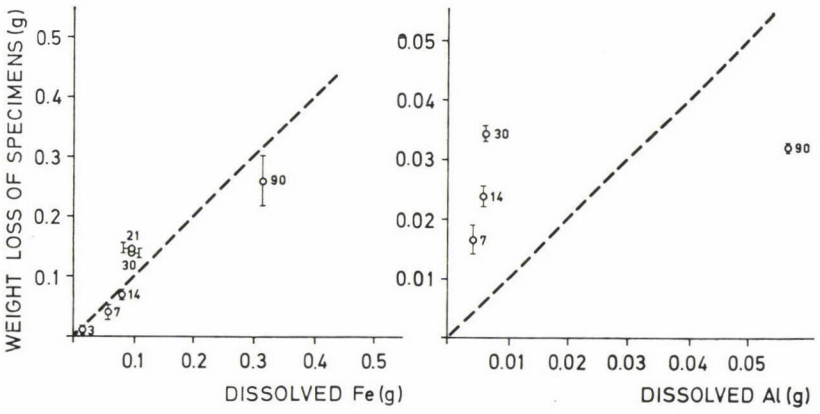

Fig. 13. Comparison of the weight loss of (5) carbon steel or (5) aluminium specimens exposed to meat pickle and the total amount of iron or aluminium dissolved in the pickle, as a function of time. The dashed line represents the correlation to be expected with the weight loss of metal (Fe or Al) specimens, the numbers show the time of exposure (days) and the vertical bars the standard deviation

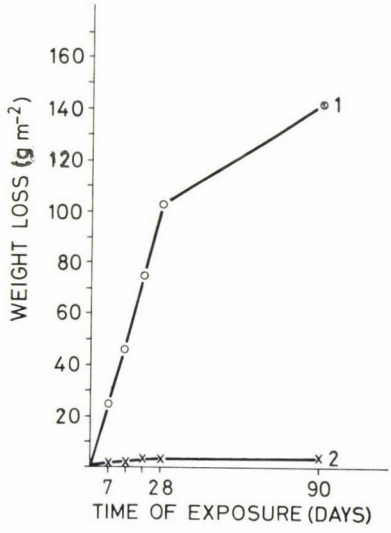

Fig. 14. Weight loss of carbon steel (1) and aluminium (2) specimens in sour-cherry juice

Fig. 15 shows the iron and aluminium content, resp., in the sour-cherry juice used as corrosive medium, compared to the weight loss of the test specimens.

The iron content in the sour-cherry juice was determined by atom absorption spectrophotometry as well. Results are shown in Fig. 15.

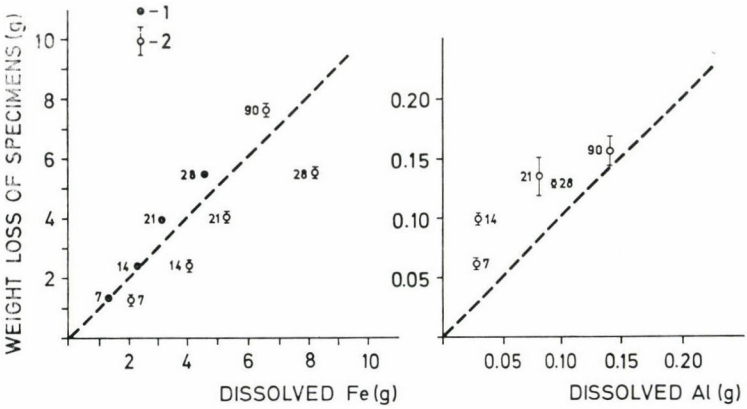

Fig. 15. Comparison of weight loss of (5) carbon steel or (5) aluminium specimens exposed to sour-cherry juice and the total amount of iron or aluminium dissolved in the juice, as a function of time. The dashed line represents the correlation to be expected with the weight loss of metal (Fe or Al) specimens, the numbers show the time of exposure (days) and the vertical bars the standard deviation. 1: dissolved Fe was determined by atomic absorption measurements; 2: dissolved Fe was determined by the method given in HUNGARIAN STANDARD (1962a)

3. Conclusions

Results of the experiments permit of the following conclusions. The corrosion rate of carbon steel in food media changes with time. In the initial phase the corrosion rate is higher and its change is more rapid, after 30 days it slowly becomes stationary and approaches a limit value. Thus, if we aim at a theoretical study of carbon steel as a construction material we have to make a detailed study of the changes occurring in the initial phase of corrosion. On the other hand if we wish to provide practical data to the construction of containers the results obtained in experiments extended over 30 days are more useful. The utilization of results obtained in experiments extending over 30 days is indicated by the fact that the exposure time of equipment used in the food industry may exceed 30 days and a juice is kept frequently well over 30 days in a storage tank.

Aluminium is substantially more resistant to food media than carbon steel. In the experiments here discussed the weight loss of carbon steel specimens was about 2–50 times higher than that of aluminium specimens.

The corrosion rate of aluminium in media containing *i. a.* chloride ions showed higher and lower values alternately. In acid media containing also halogen ions the mechanism of aluminium corrosion was different, conditions for pitting corrosion were present. Thus in judging aluminium corrosion pitting, spot corrosion and the formation of grooves have to be taken into account (Fig. 3).

The weight loss of the specimens and the metal content in the aggressive medium did not show a direct correlation, particularly in the case of aluminium. This is due probably to the lower precision of aluminium determination. Therefore the metal concentration in the food medium does not permit of drawing conclusions as to the corrosion of aluminium or carbon steel as was suggested by KOCH and BREKER (1958). The results of metal determination depend on the method applied and results obtained by different methods are not always comparable.

Thus to obtain a complete picture of the corrosion of metals in food media it is essential to test the corrosion of the construction material and the metal contamination of the food medium, as well.

These experiments, started with a completely different aim, are considered preliminary experiments. They raised a number of interesting problems and the continuation of the study, extended over other metals and food media, seems very useful.

*

I am indebted to Mrs. G. SZABÓ (RESEARCH INSTITUTE FOR VITICULTURE AND ENOLOGY) for the performance of the atom absorption measurements. Thanks are due to Misses K. LIKTOR, K. PERÉDI and É. ZSARNAY for their devoted technical assistance.

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KEEPING QUALITY OF CELERY VARIETIES

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Three celery varieties: *Hegykői*, *Frigga* and *Imperator* were stored in cases in a cellar of 0–11 °C and of 70–85% relative humidity. A part of variety *Hegykői* was stored in polyethylene bags (thickness 0.2 mm) with the other samples. During the 9-week storage period weight loss, withering and consistency of the samples were studied. During this period microbiological spoilage was not observed.

For the control of withering a new method was tested based on the measurement with sliding caliper of the diameter of the celery head at its widest point and on the χ^2 test of the frequency distribution of diameters.

The results may be summarized as follows.

— Withering and weight loss was highest in variety *Hegykői*, stored in cases. This sample was also the softest.

— The keeping quality of varieties *Frigga* and *Imperator* was found to be better than that of *Hegykői*, when stored in cases.

— Storage in polyethylene bags proved very satisfactory during the 9-week test period. This test was carried out only with variety *Hegykői*. Weight loss of the packaged sample was very low, it amounted to 3.4% during the 9-week storage period. Withering was not observed. The consistency of the samples hardly changed at all.

Because of the seasonal character of fresh fruit and vegetables it is very important to increase their storage life by adequate methods of storage. In view of the demands of the consumers and the limitations of storage facilities, the processing industries play a substantial role in the provision of the consumers with preserved goods. Thus it is of extreme importance to select varieties which are suitable for both storage and processing.

The aim of our study was to compare some celery varieties as regards suitability for storage.

To provide appropriate storage conditions, *i. a.* the knowledge of the following data is necessary.

1. Variety
2. Conditions of growing
3. Method of harvesting (manual or mechanical).

Characteristics of variety, resistance to diseases affect not only cultivation but the efficiency of storage, as well. Opinions differ as to the effect of growing conditions upon keeping quality. HÁJAS (1972) found that the keeping quality is affected by watering the plant during cultivation, by the rainfall in the year of cultivation, the structure and supply of nutrients in the soil. Fertilizers do not seem to affect keeping quality (ZIEGLER & BÖTTCHER,

1966). However, nitrogen fertilization is an exception, because it increases enzyme activity in the plant and unfavourably affects the moisture-retaining capacity of the cells.

Experience so far has shown a temperature of 1–4 °C and a relative humidity (RH) of 90–95% most suitable for the storage of root vegetables (ZIEGLER & BÖTTCHER, 1966; BESLER, 1970; LINDSAY & NEALE, 1971; CRISP, 1972; HÁJAS, 1972; SIEDEL *et al.*, 1972; LÉPOLD, 1973; SMITH, 1967; ERNEST, 1972).

LINDSAY and NEALE (1971) found that the maximum keeping time of celery is 6–10 weeks. The international standard ISO/TC 34 556 E specifies a maximum of 12 weeks storage period for celery.

Storage experiments were carried out at the RESEARCH STATION, FERTŐD, OF THE INSTITUTE FOR HORTICULTURAL RESEARCH (HÁJAS, 1972). The celery was stored in cases, in wooden containers and plastic bags. The cases and wooden containers were covered in part with plastic film. The storage temperatures were 1 and 4 °C. According to the results the best conditions for the storage of celery are: the heads without washing are packaged in plastic bags and kept at 1 °C.

According to STOLL (1971) the most favourable conditions for the storage of carrots and celery in a controlled atmosphere are: an oxygen content of 3%, 3% of CO_2 and 94% of N_2. If the CO_2 content of the atmosphere increases above the specified level the spoilage of the vegetables accelerates.

PRINCE (1971) stored carrots, celery, cauliflower, parsley and potatoes at 1 and 15 °C, resp. He aimed at establishing the time and place of the attack of infection. He found that the vegetables arrived at the place of storage already infected. Infection seemed to be less intense when celery was planted out later. This phenomenon is probably dependent on the climatic conditions.

The aim of the present study was to select a suitable variety and the comparison of storage in open cases and in polyethylene bags.

A new method was tested for following up withering. The results were compared to data on weight loss and consistency.

1. Materials and methods

1.1. Raw materials

The three celery varieties were obtained from the RESEARCH STATION, FERTŐD, OF THE INSTITUTE FOR HORTICULTURAL RESEARCH, 150 to 200 kg of each variety. The celeries were cultivated on medium-bound clay soil. The soil was fertilized with stable-dung (300 metric centner per hectare) in 1972 and with chemical fertilizer (400 kg/hectare superphosphate, 400 kg/hectare potassium and 150 kg/hectare nitrogen) in 1973.

The vegetables were received on November 28, 1974. They were harvested by hand a few days earlier. On receipt they were extremely muddy and damp, thus, they were spread in a thin layer and stored for about 2 weeks. The work was started on December 9, 1974. This date is considered 0 time in the experiments.

The varieties used were: *Hegykői*, *Frigga* and *Imperator*. BECKER and co-workers (1973) characterized these varieties as follows:

Variety *Hegykői* (*Apium graveolens* L. var. *rapaceum*) suitable for consumption in the fresh state and for storage. It may be cultivated on a large scale and in the garden. It has a long growth season. It is easy to clean for sale or for winter storage since it has few roots on the head. Its foliage may be used for the extraction of essential oil or for drying, however the mass of foliage is smaller than with other varieties. Its nutrient requirement is high. The water requirement is high, it needs watering in dry weather. It needs not be picked early because it resists well the early autumn frost of -4 to -5 °C, while it develops substantially in the final growth period. The growth period comes to an end when the lower leaves turn yellow. It is resistant to disease and not prone to hollowing. On blanching it becomes white. Morphology of the variety: medium height, thick foliage. Short leaf-stalks, erect leaves of dark green colour. The head is spherical, of medium size with smooth surface. The roots are thin and grow on the lower side of the head. The flesh is white and compact.

Variety *Frigga* (*Apium graveolens* L. var. *rapaceum*) suitable for large scale and domestic cultivation. It may be consumed fresh, stored or processed.

It is reliable to give a rich yield, even when not watered, therefore it can be cultivated profitably.

Morphological characteristics: of little or medium foliage, medium strong growth. The head is elongated round, medium-sized, slightly larger than that of variety *Hegykői*, of compact white consistency with smooth surface, frequently showing green discoloration. The flesh is medium compact, fairly solid, white with beige marbling. It is prone to hollowing. Thick, strong roots on the lower side, few on the sides.

Variety *Imperator* (*Apium graveolens*, L. var. *rapaceum*) suitable for cultivation on a large scale and in the garden, and for storage. Economical even when planted out in June, reliable, giving a rich yield. Foliage surface fairly large, may be used for drying.

Of medium long growth period, sound variety, resistant to disease.

Morphological characteristics: medium foliage with some very large leaves on medium strong stalks. The surface of the leaves is slightly rough. The head is large and spherical with rough surface. The roots grow on the broadened bottom of the head. The flesh is yellowish white, with tiny mauve spots, of medium compactness, prone to hollowing, slightly spongy.

1.2. Storage

The selected heads were placed in wooden cases of $40 \times 60 \times 35$ cm. Conditions of storage were 9–11 °C and 70–85% RH. A part of variety *Hegykői* was stored in polyethylene bags, containing 30 heads each, of a total weight of 3–4 kg. The bags were tied up and stored for 9 weeks.

For the test for withering the celery heads were nest-packed in cases of $40 \times 60 \times 12$ cm ($N = 37$ pieces, 5–10 kg, dependent on variety). Nestpack was used to prevent mixing up of heads when measured. In nestpack the heads could not infect each other.

1.3. Weight loss

The weight of each case was established at predetermined points of time (0, 5 and 9 weeks). Loss of weight was expressed as percentage of the original weight. Each case contained about 8–10 kg celery head, three cases of each variety.

1.4. Withering test

Celery heads were nest-packed, 37 pieces of each variety. At predetermined points of time the widest diameter of the heads was established by the use of a sliding caliper. The values thus obtained were expressed as percentage of the initial diameter and this was understood to be the "withering %". The frequency distribution of withering percentage values was determined and the results were compared by the χ^2 test.

1.5. Determination of consistency

A *Texturometer* (ZENKEN CO., Japan) was used to determine the consistency according to KOVÁCS and VAS (1969). Slices of 12 mm thickness were cut from the celery heads and consistency was measured near to the skin ($N = 5$).

2. Results

2.1. Weight loss

The weight loss of the different celery varieties is illustrated in Tables 1 and 2 and Fig. 1.

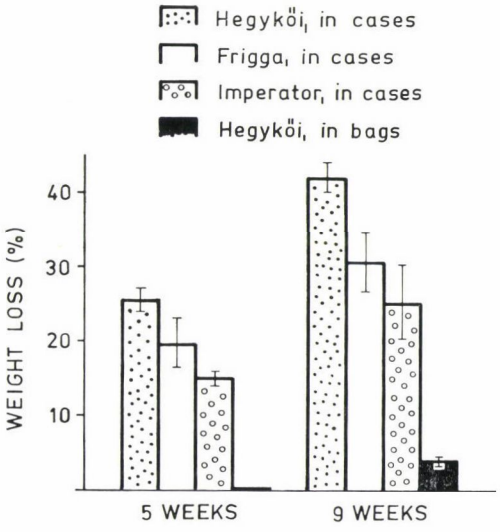

Fig. 1. Weight loss in different celery varieties as a function of time (Storage temperature: 9–11 °C; relative humidity: 70–85%. RH for celery stored in bags: ~ 100%)

Table 1

Weight loss in different celery varieties, stored in various ways, as a function of time

Variety	Weight loss at 9–11 °C (average and standard deviation as % of the initial weight)			
	In a space of 70–85% RH		≤ 100% RH	
	stored in cases		stored in polyethylen bags	
	storage time (weeks)			
	5	9	5	9
Hegykői	25.6±1.45	42.2±1.68	0±0	3.4±0.99
Frigga	19.4±3.40	30.6±4.09		not studied
Imperator	14.8±0.65	25.3±4.88		not studied

The greatest loss was observed with the variety *Hegykői*, the loss of variety *Imperator* was the lowest. Variety *Hegykői* suffered a greater loss when stored in cases than packaged in bags.

A loss of 10% was reached with variety *Hegykői* in 2 weeks, with *Frigga* in 2.7 weeks and with *Imperator* in 3.3 weeks; 20% loss was reached by variety *Hegykői* in 4 weeks, with *Frigga* in 5.3 weeks and with variety *Imperator* in 7 weeks. The loss of variety *Hegykői*, when stored in polyethylene bags, did not reach 10% during the experimental period of 9 weeks.

After a storage period of 5 weeks test for significance has shown variety *Hegykői* to be of the lowest keeping quality ($P \geq 95\%$) in comparison with

Table 2

Test of significance of differences between weight loss data (t values)

Variety	*Hegykői* in bags	*Frigga* in cases	*Imperator* in cases
			5 weeks
Hegykői, in cases		2.91*	11.82***
Hegykői, in bags+			
Frigga, in cases			2.30
			9 weeks
Hegykői, in cases	38.50***	4.53**	5.67**
Hegykői, in bags		11.30***	7.61***
Frigga, in cases			1.44

+ Variety Hegykői did not loose weight during 5 weeks storage when stored in bags
N = 3 cases or bags
$t_{95\%}$ 2.57*
$t_{99\%}$ 4.03**
$t_{99\%}$ 6.86***
* The samples differ on a probability level of 95%
** The samples differ on a probability level of 99%
*** The samples differ on a probability level of 99.9%

Frigga ($P \geq 95\%$) and *Imperator* ($P \geq 99.9\%$). There was no significant difference in weight loss between varieties *Frigga* ($P \geq 95\%$) and *Imperator* ($P \geq 99.9\%$). There was no significant difference in weight loss between varieties *Frigga* and *Imperator*.

After 9 weeks storage variety *Hegykői*, packed in polyethylene bags was found to be significantly better than all samples stored in cases. The weight loss of this sample was the lowest. The difference was significant at probability level 99.9%.

Of the samples stored in cases the loss in variety *Hegykői* was higher than that in *Frigga* or *Imperator* ($P \geq 99\%$). No statistical difference was found in the weight losses of the latter two samples.

2.2. *Withering*

The diameter of celery heads was periodically controlled during the test period. The values thus obtained were expressed as percentage of the initial diameter and designated as withering percentage. The frequency distribution of these values is shown in Fig. 2.

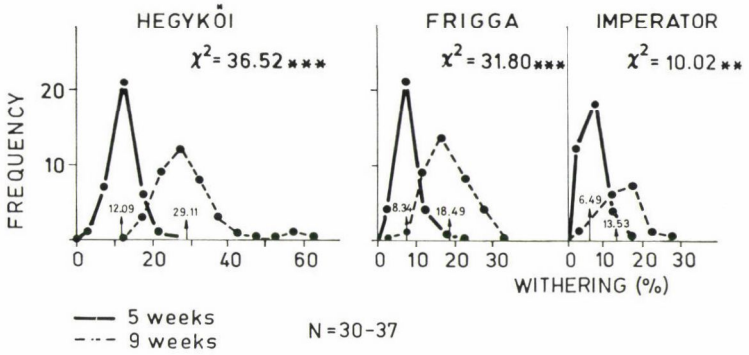

Fig. 2. Withering of varieties: *Hegykői*, *Frigga* and *Imperator* after 5 and 9 weeks of storage (N = 30–37)

The highest withering percentage was found in variety *Hegykői*, stored in cases. The frequency distributions of withering percentages as measured after 5 and 9 weeks of storage showed significant difference in all three varieties. The difference was significant at the probability level of 99.9% with varieties *Hegykői* and *Frigga* and at 99% probability with *Imperator*, when all the samples were stored in cases. The significance test between varieties (Table 3) showed variety *Hegykői* to suffer a significantly higher withering ($P \geq 99.9\%$) than *Frigga* or *Imperator*.

Table 3

Significance of differences between withering values (χ^2 values) of celery varieties stored in cases (N = 65–72)
(independent of diameters, in the 5–9 cm range)

	Frigga	*Imperator*
	5 weeks	
Hegykői	10.75***	10.84***
Frigga		2.34
	9 weeks	
Hegykői	16.69***	26.68***
Frigga		2.09

*** The samples differ on a probability level of 99.9%

Varieties *Frigga* and *Imperator*, packed in cases, did not differ when tested after 5 weeks or 9 weeks. Withering was not observed with variety *Hegykői*, when packed in polyethylene bags.

2.3. Change in consistency

Consistency was established on celery slices of 12 mm thickness. Results are given in Fig. 3 and Tables 4 and 5.

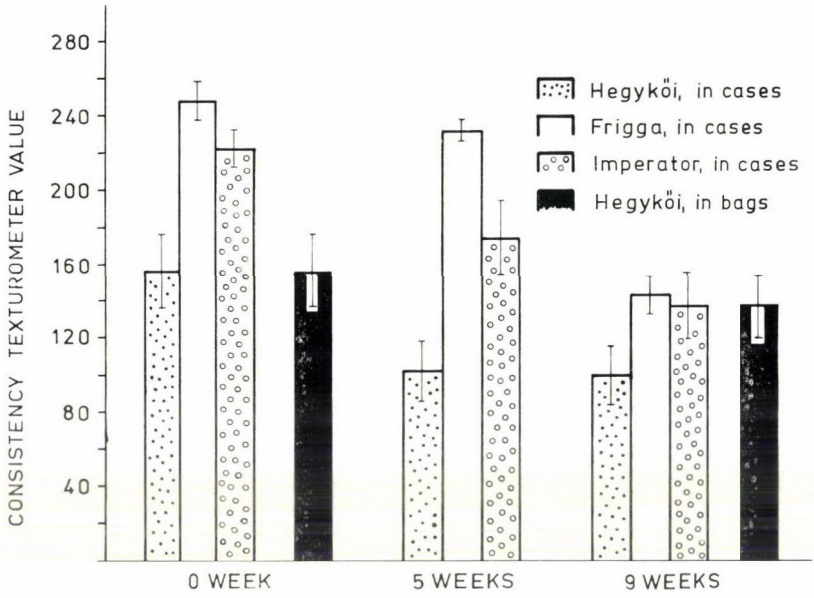

Fig. 3. Consistency of the different celery varieties as a function of time (N = 5)

Table 4

Change in the consistency of the celery varieties as a function of storage time

Variety	Change in texture at 9–11 °C (Texturometer degrees)			
	Stored in cases at 70–85% RH			in bags
	Storage time (weeks)			
	0	5	9	9
Hegykői	(3) 156.67±13.12	(5) 103.3±15.78	(5) 101.0±16.01	(4) 138.5±14.90
Frigga	(3) 248.00±10.35	(5) 232.2±6.23	(5) 143.4±9.43	not studied
Imperator	(3) 222.17±9.72	(5) 174.0±21.37	(5) 138.9±18.28	not studied

N = 3 or 5, resp.

The consistency of the samples weakened during the storage period. Variety *Hegykői* softened most extensively, when packaged in cases, while *Frigga* remained hardest. However, the consistency of the variety *Hegykői* hardly changed at all when stored in polyethylene bags.

Table 5

Test of significance of differences between the consistency values of the different celery varieties (t values) (Measurements were carried out on the surface of celery)

Variety	*Hegykői* + in bags	*Frigga* in cases	*Imperator* in cases
	5 weeks		
Hegykői, in cases		4.41**	3.21**
Hegykői, in bags			
Frigga, in cases			2.35*
	9 weeks		
Hegykői, in cases	3.60**	4.56**	3.12*
Hegykői, in bags		0.61	0.04
Frigga, in cases			0.44

+ Variety *Hegykői*, stored in bags, was measured on 0 day and after 9 weeks of storage N = 5, except *Hegykői*, in bags, where N = 4
* The samples differ on a probability level of 95%
** The samples differ on a probability level of 99%

After 5 weeks of storage the significance test has shown *Hegykői* in cases to be softer ($P \geq 99\%$) than *Frigga* or *Imperator* stored in cases. *Frigga* was found harder than *Imperator*.

After 9 weeks of storage variety *Hegykői* was shown to be softer than *Frigga* ($P \geq 99\%$) and *Imperator*, when all three samples were stored in cases. Difference was not observed between *Frigga* and *Imperator*. Variety *Hegykői* was found to have a better consistency when packaged in bags than in cases ($P \geq 99\%$) and did not differ from varieties *Frigga* and *Imperator*, packaged in cases.

In Figs. 4, 5 and 6 the results of the correlation tests are shown.

The correlation between weight loss and change in consistency is close ($r = 0.87$), while between weight loss and withering the correlation is very close ($r = 0.99$). A close correlation was found to exist between the extent of withering and the consistency values ($r = 0.88$).

It is shown by the correlation tests that consistency and withering are highly affected by weight loss.

The simple new method, applied for testing withering was found suitable for establishing the extent of withering, results thus obtained form a satisfactory characteristic of the consistency of celery varieties as proven by the correlation tests.

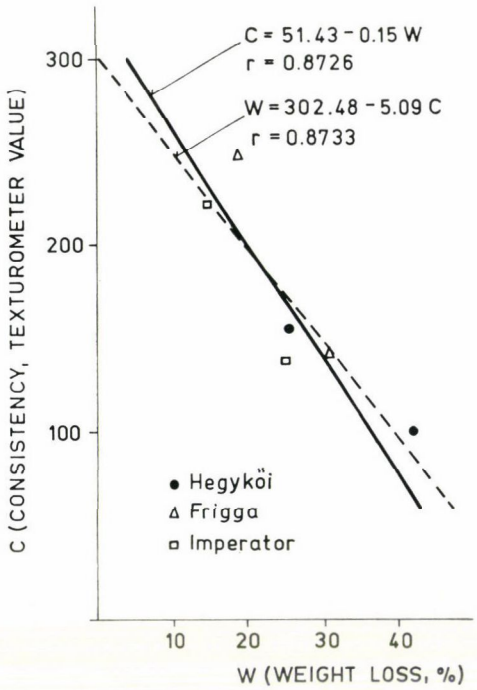

Fig. 4. Correlation between weight loss and change in consistency in celery varieties: *Hegyköi*, *Frigga* and *Imperator*. (Each point in the Figure marks the average of 3–5 individual values)

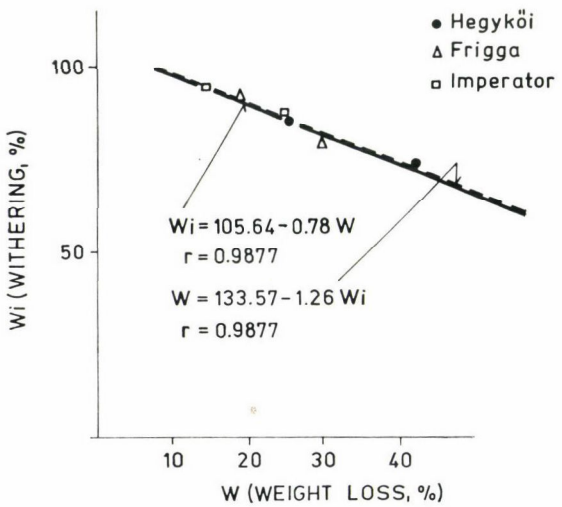

Fig. 5. Correlation between weight loss and withering in different celery varieties. (Each point in the Figure indicates the average of 15–37 withering percentage values or 3 weight loss values)

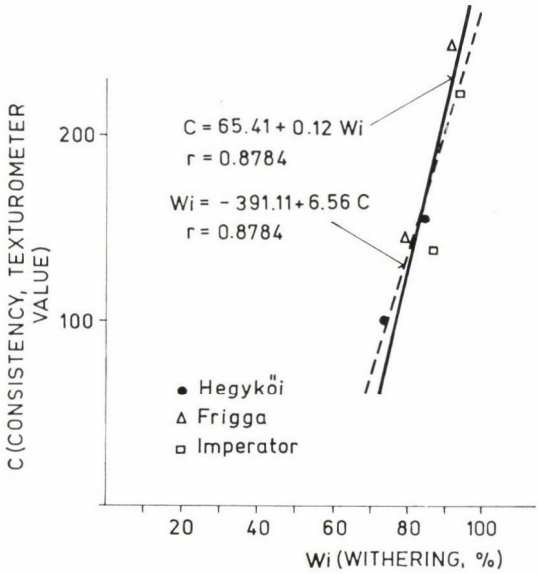

Fig. 6. Correlation between consistency and withering values. (Each point in the Figure represents the average of 15–37 withering values or 3–5 consistency values)

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COMPARATIVE STORAGE STUDIES ON FUMIGATED AND IRRADIATED WHEAT

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In semi-pilot scale studies, the effects of fumigation and gamma radiation on microbial growth and baking properties of stored (up to one year) wheat, have been investigated. Under identical conditions of storage, either indoor or outdoor, at least two to three fumigation treatments per year are required to keep the insect population within limit, whereas, irradiation only once at disinfestation dose (30 krad) is sufficient. The fumigant (aluminium phosphide) has no deleterious effects on the rheological properties and baking qualities of wheat, stored up to eight months. The present data also indicate that irradiation, at moderate dose, does not impair breadmaking quality of stored wheat.

The losses during storage of food grains in tropical countries are immense due to adverse climatic conditions of high humidity and temperature, which accentuate the proliferation of spoilage microorganisms and insects. While no precise estimates are available, it is possible that the destruction of total food produced in India could be as high as 30–35% (Nair & Brownell, 1965) including loss by rodents, compared to average world loss of 20%, as estimated by FAO (Cornwell, 1964).

The use of chemical fumigants is the conventionally most adopted measure for insect control (Schultz & Lee, 1966). In India, wheat is generally stored in grain silos, storage bins, large and small or in centralized warehouses with stacked *hessian* bags. Stored grain is subjected to fumigation treatment from time to time, at intervals often arbitrarily determined. However, factors such as inability to destroy all the granary insect species present in various development stages (Cornwell & Bull, 1960), uneven penetration power and the problem of health hazards due to toxic residues (Disney & Fowler, 1974a), preclude fumigation as a method of choice.

Application of ionizing radiations such as electrons and gamma rays at low dose levels [20–30 krad (0.2–0.3 kGy)] has been considered to be a promising method not involving use of chemicals for insect control in stored wheat and wheat products (Mayer, 1969). Irradiation effectively destroys all metamorphic stages and sterilizes the adults of about thirty known species of granary insects (Hassett, 1956). Besides, there is no residual effect, although certain subtle compositional changes in wheat starch (Ananthaswamy *et al.*, 1970), lipids (Tipples & norris, 1965) and proteins (Doguchi, 1969; Srinivas *et al.*, 1972) have been reported. However, information on the possible effects on the quality of wheat after long storage is incomplete.

The present investigation reports the comparative results on the changes in temperature, moisture content, and proliferation pattern of spoilage organisms, during prolonged storage of untreated, irradiated or fumigated wheat. Results on the visco-elastic properties and baking quality of the wheat samples, withdrawn at various time intervals, are also included. The acceptability of leavened and unleavened Indian bread *(Chapati)* prepared from these samples has been ascertained by known subjective methods.

1. Materials and methods

About 15 tons of newly harvested *Punjab Dara* variety of wheat were procured from FOOD CORPORATION OF INDIA, and divided into 9 lots. Three lots were kept as they were and served as control. The remaining six lots were deliberately infested with known numbers (2000/bin) of newly emerging grain pest, *Calandra oryzae*, prior to either radiation or fumigation treatment. Aluminium phosphide tablets (marketed as *Cephos* by EXCEL INDUSTRIES, Bombay), were purchased from local market.

1.1. Irradiation

Additionally infested grains were irradiated at 30 krad dose level, adequate for efficient disinfestation (CORNWELL & BULL, 1960) in a through-flow ^{60}Co grain irradiator [ATOMIC ENERGY, CANADA, Ltd., 28 kCi (1.036 PBq) with a dose variation of 30%]. This is specially designed for cereals, with throughput adjusted to 230 kg h^{-1} at a dose of 15 krad. The grains were passed twice through the irradiator and directly collected into storage containers, by specially designed rotorlift and conveyor mechanisms.

1.2. Fumigation

Fumigation of additionally infested wheat was carried out immediately and at intervals of four months thereafter. Aluminium phosphide (3 g/ton of wheat), widely used as a fumigant in India for bulk storage of wheat and other cereals, was applied for an exposure time of 5 days. Treated wheat samples were then aerated to remove excess fumigant and stored.

1.3. Storage

Wheat samples were stored in corrugated metal bins (capacity 1600 kg each), specially designed to be gas-tight and insect-proof. Two bins each of control, irradiated or fumigated samples were kept indoor, under ambient conditions [27 ± 3 °C and 55% relative humidity (RH)] and one of each was

kept outdoor under atmospheric conditions (23–38 °C; 40–85% RH), for about 12 months. The bins were well protected from the monsoon rains. First sampling was done 2 months after the initiation of the experiment and subsequent samplings were at intervals of 1 month. Samples (0.5–0.8 kg) were drawn with a probe inserted through the lid into wheat columns from three different layers. The temperature and relative humidity inside the bins were also recorded. The samples were pooled, mixed thoroughly and used for various analyses. Sufficient precautions were taken to prevent infestation from atmospheric exposure. In untreated wheat samples, insects began emerging within four months and, therefore, only consumable portions were used for examination.

Moisture content was determined by air oven method (103–104 °C) (POMERANZ & MELOAN, 1971). Temperature was recorded and for the assessment of the degree of infestation, number of weeviled grains were counted.

1.4. Total bacterial and mold counts

Samples of wheat were crushed and suspended in saline solution and serial dilutions prepared. The pour-plate method was employed for the propagation of cultures. Known aliquots were plotted on potato-dextrose agar, pH 5.2 (DIFCO) for total mold counts and on nutrient agar (pH 7.0) for total bacterial counts. The plates were incubated for 72 and 48 h, resp., at 30 °C. Different colonies formed, were counted and total bacterial and mold counts expressed per g of wheat.

1.5. Rheological properties

For assessment of the rheological properties of stored grains, samples pooled at 4 months interval, were ground in a *Buhler*'s experimental mill at extraction rate of 65–70%, before use. Gelatinized viscosity at constantly increasing temperature was measured in *Brabender Amylograph* (Au). Water absorption capacity and dough development time to obtain 500 *Brabender* units (BU) were measured in *Brabender Farinograph*. Drop in dough consistency was determined 12 min later (with continuous mixing). These two values determined the mixing properties of wheat gluten. The behaviour of the relaxed dough towards continuous mechanical action was recorded after resting for 1 h (KENT-JONES & AMOS, 1967).

1.6. Bread making

Baking test for leavened bread was carried out according to standard straight dough lean formula (MATZ, 1960). Ingredients used were: wheat flour (100 g), hydrogenated oil (4.0 g), salt (1.5 g), sugar (4.0 g) and dry yeast (4.0 g),

Water (about 62 ml) was added as required to obtain optimum dough consistency of 500 *Brabender* units (BU). The dough was fermented (2 h at 30 °C), proofed (1 h) and loaves were baked for 25 min, at 219 °C. Loaf volume was measured by seed displacement method. Bread crust was extracted with 70% ethanol and the intensity of colour measured at 460 nm in *Bausch* and *Lomb Spectronic* 20.

Indian unleavened bread *(Chapati)* was prepared by conventional method. The dough, obtained from wheat flour with addition of 60–65% water, 1.0 salt and 10% hydrogenated oil was kneaded, then divided into small balls each weighing about 35 g) and rolled evenly on a wooden board with a rolling pin. *Chapati* (6″ in diameter) was then baked on a hot plate for 1–2 min.

1.7. Sensory evaluation

Acceptability of leavened and unleavened breads was evaluated by a taste panel, consisting of 12 trained members of the scientific staff. A modified triangular test with a nine point numerical hedonic scale was used. Off-characteristics such as off-odour, discoloration, irradiation flavour, texture, *etc.*, were judged by using 10 point intensity scale.

2. Results

2.1. Infestation in stored grains

The average % of weeviled grains in the stored samples is shown in Fig. 1. A progressive increase with storage time in untreated samples (up to 32%) and to a lesser extent (6.5%) in fumigated samples is observed. In irradiated samples, the increase in weeviled grain is negligible.

2.2. Physical properties and bacterial counts

Average moisture content and temperature changes in control (untreated), fumigated or irradiated wheat samples, stored for 12 months, indoor or outdoor are plotted in Figs. 2 and 3. Increase in moisture content, varying between 1–5% was observed in samples, stored outdoor or indoor during the experimental period of 12 months. Similarly, temperature of outdoor samples was raised during the months of October and May due to seasonal variations. It can be seen that total microbial population and mold counts rapidly declined (by about 1–2 log cycles), soon after irradiation and remained more or less constant throughout the experimental period; in fumigated samples, however, increased mold growth was evident at 4 months, and was stabilized after second fumigation. Mold outgrowth increased significantly in control (untreated) wheat, stored indoor or outdoor, for more than 8 months.

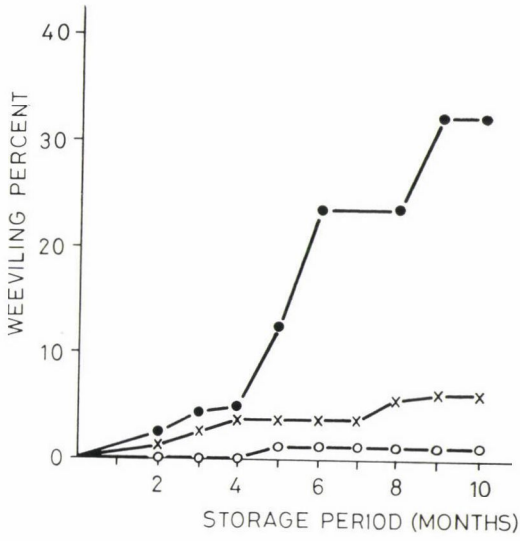

Fig. 1. Rate of infestation in stored grains – weeviled grains from different samples were counted at random and expressed as % of total seeds. ●——●, untreated; ○——○, irradiated; x——x, fumigated

Table 1

Evaluation of farinogram

Wheat flour (300 g) was taken in Farinograph mixing chamber (30 °C) and water was added till a dough of 500 BRABENDER units (BU) consistency was obtained. Dough properties (listed below) were evaluated from the "normal titration curve" as described in the text

Parameter	Control	Irradiated	Fumigated
Dough development time (min)			
Initial	6.5	8.5	7.5
4 months	7.5	7.5	7.5
8 months	7.25	5.75	5.0
Drop in dough consistency (BU)			
Initial	90	120	110
4 months	120	120	120
8 months	100	100	100
Dough stability (BU)			
Initial	20	40	40
4 months	40	40	40
8 months	40	40	40

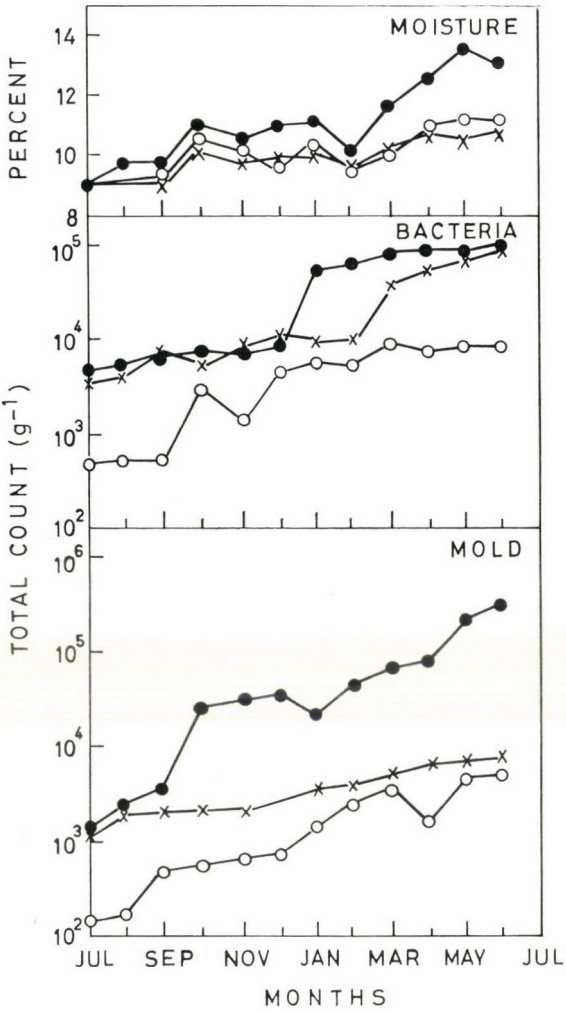

Fig. 2. Effect of indoor storage on physical properties and spoilage pattern of wheat. Wheat samples, untreated, irradiated or fumigated, were stored for 12 months under controlled ambient conditions (25 ± 3 °C; 55% RH). Samples were examined at different time intervals. ●——●, untreated; ○——○, irradiated; x——x, fumigated

2.3. Rheological properties

The comparative data on the rheological properties of stored wheat samples are summarized in histograms (Figs. 4 and 5). The results obtained at 4- and 8-month intervals were essentially the same and hence, only the later ones are presented. After 8 months, the untreated samples were not fit for human consumption due to rapid outgrowth of mold and were, therefore, not tested for rheological properties. It can be seen that (Fig. 4) the initial

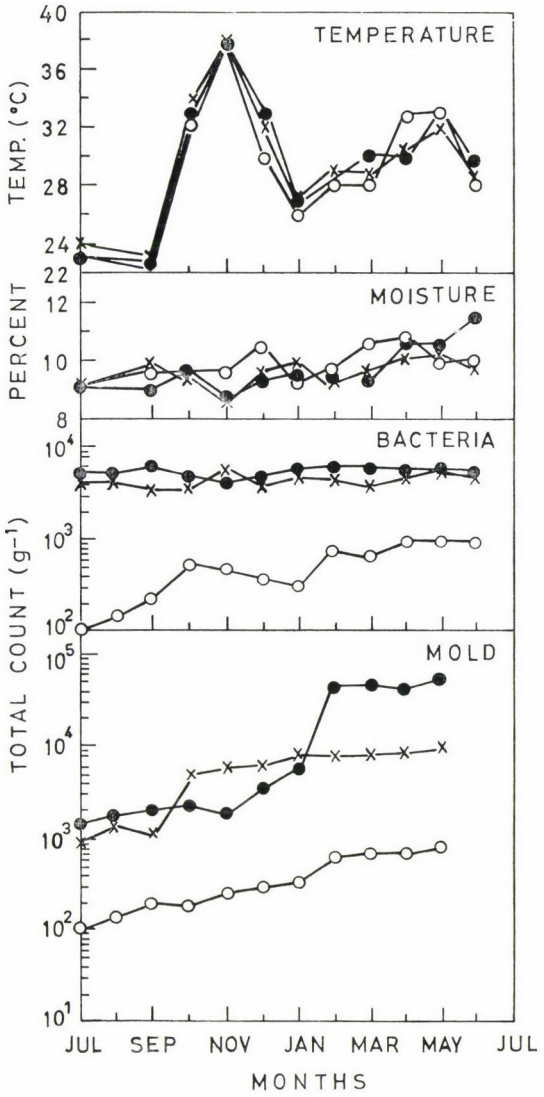

Fig. 3. Effect of outdoor storage on physical properties and spoilage pattern of wheat. Wheat samples were stored for 12 months in atmospheric conditions, varying in temperature (23–35 °C) and relative humidity (40–85% RH). Samples were withdrawn at indicated time intervals. ●——●, untreated; ○——○, irradiated; x——x, fumigated

maximum gelatinization viscosity was low in irradiated samples (800 AU), compared to control (940 AU). This dropped significantly on storage in all the samples. Water absorption capacity of samples was not affected by fumigation (Fig. 5); however, slight increase (about 10%) was noted in irradiated samples on storage.

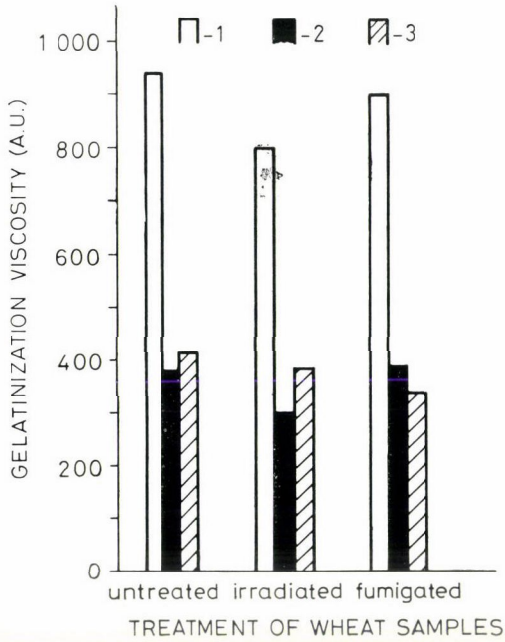

Fig. 4. Changes in maximum gelatinization viscosity of wheat during storage. Wheat flour (80 g) was mixed with 450 ml of water to form a lump-free suspension and heated in *Amylograph* revolving container with a steady rise of 1.5 °C min^{-1} until complete gelatinization occurred. The values, in terms of amylogram units (AU), are plotted in the histogram. 1: initial; 2: stored indoor for 8 months; 3: stored outdoor for 8 months

Results obtained with *Farinograph* were evaluated to characterize the dough properties (Table 1). Dough development time was reduced on storage of irradiated and fumigated wheat. Dough stability was low in control, but on storage, it reached maximum level. It can be noticed that storage conditions indoor or outdoor had negligible effects on the rheological properties of untreated, irradiated or fumigated samples.

2.4. Baking properties

Studies on baking properties and organoleptic evaluation were carried out only with wheat samples stored indoors. Since storage conditions did not change rheological properties, no variations in their baking properties could also be expected. It can be seen that the specific loaf volume was very low initially in all the samples (Table 2). This improved on storage, but to a greater extent in treated samples ($P \leq 0.05$) compared to untreated control. Crust colour of the breads was also better in stored samples. These were reflected in sensory evaluation. The breads prepared initially (before storage), scored low on hedonic scale, because of uneven loaf, non-uniform cell structure and

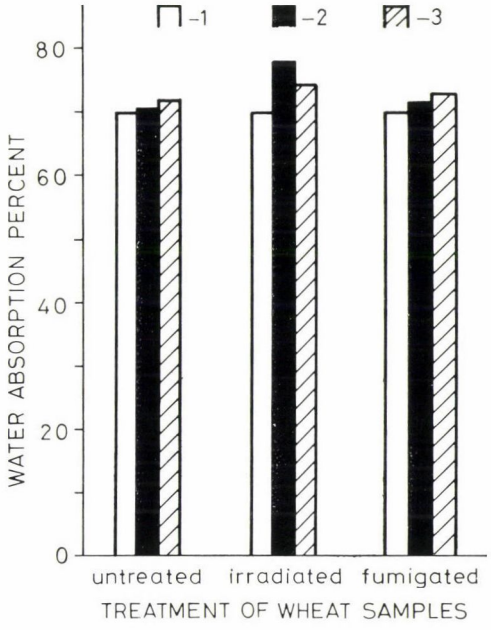

Fig. 5. Water absorption capacity of stored wheat. The histogram shows the water absorption capacity of untreated and treated wheat, stored indoor or outdoor for 8 months. 1: initial; 2: stored indoor for 8 months; 3: stored outdoor for 8 months

slight discoloration. But those prepared from stored wheat were highly acceptable ($P \leq 0.001$) as these external characteristics were improved. Similarly, no off-characteristics such as off-flavour, irradiated flavour, *etc.*, were recorded as inferred from the evaluation of breads by panel members, using 1–10 points intensity scale (Table 2).

Acceptability of non-leavened Indian bread was not affected by radiation or fumigation treatment. In initial irradiated samples, slight discoloration was noticed which had disappeared in stored samples. The acceptability of *Chapaties* prepared from irradiated, stored wheat was significantly better ($P \leq 0.05$) compared to initial samples.

3. Conclusions

Though fumigants are toxic to most insect species, some of the commonly infesting ones in wheat, *e.g.* figmoth and khapra beetle are resistent (CORNWELL & BULL, 1960). Lack of emergence of weevils in irradiated samples (Fig. 1), even at 10 krad (RAHALKAR & LEWIS, 1969), demonstrates the effectiveness of irradiation; Under Indian Standards (FOOD CORPORATION OF INDIA), grains weeviled up to 5% are permissible. Inhibition of mold growth (MAJUMDER *et al.*, 1965; RAGHUNATHAN *et al.*, 1969) and formation of mycotoxins such as

Table 2

Effect of irradiation, fumigation and storage on the quality and acceptability of breads
Breads were baked from wheat samples, immediately after treatment and after 8 months storage. These were evaluated for external (loaf volume, crust colour) and internal (colour, texture *etc.*,) characteristics as described in the text

Bread Quality	Treatment of wheat					
	Untreated		Irradiated		Fumigated	
	Initial	Stored	Initial	Stored	Initial	Stored
Leavened bread						
Specific loaf volume cc/g	2.34	*2.64	2.34	*3.16	2.33	*3.33
Crust colour % transmission	85	95	78	96	86	96
Mean score on hedonic scale	5.0 ± 0.22	***8.0 ± 0.54	4.5 ± 0.30	***8.0 ± 0.28	5.0 ± 0.35	***8.5 ± 0.33
Discoloration on intensity scale	1.0	1.0	1.0	2.0	1.0	1.0
Unleavened bread (Chapati)						
Mean score on hedonic scale	7.61 ± 0.26	7.8 ± 0.37	7.4 ± 0.37	**8.2 ± 0.14	7.7 ± 0.28	8.0 ± 0.17
Discoloration on intensity scale	1.0	1.0	2.3	2.0	1.0	1.0

* significant ($P \leq 0.05$) difference as compared to initial
** significant ($P \leq 0.01$) difference as compared to initial
*** significant ($P \leq 0.001$) improvement as compared to initial

aflatoxin (RAO & HAREIN, 1972; VAN DE GRAFT *et al.*, 1973) have been reported for several fumigants. Increase in mold growth after the first fumigation (Figs. 2 and 3) indicates that their effect lasts only for a short period and that repeated treatments are necessary. In control samples, definite correlation has been observed between moisture content and outgrowth of microbial population. Increase in moisture content by 2 to 5% (Figs. 2 and 3) in storage bins, kept even indoor under controlled conditions, may be attributed to establishment of differential thermal gradients, mainly in tropical countries (MAJUMDER, 1970). Interactions of grain bulk, thermal properties of storage structures and temperature fluctuation may also contribute to accelerated inequilibrium in moisture distribution. Such relation for insect population also has been reported for stored rice (DOUGIAS & TULLIS, 1950) and maize (CHRISTENSTEN, 1957). Thus, irradiation has a distinct edge over fumigation treatment, since a single operation is sufficient to control mold growth.

Another problem to reckon with is that of the residual toxic effects of aluminium phosphide, when used as fumigant. It releases phosphine leaving aluminium oxide as residue (WHITNEY, 1961). Though it possesses great fugacity, rendering it easily removable by aeration, residual phosphorus

compounds such as oxyacids can still be present after prolonged operation (DISNEY & FOWLER, 1974a). Parallel chemical and radiometric determinations using ^{32}P-labelled phosphine have shown that the possibility may not exist for isotope exchange reactions between the phosphorus of phosphine and that of grains (DISNEY & FOWLER, 1974b). However, the existence of non-volatile residues cannot be ruled out when used in actual commercial situations (ROBINSON & BOND, 1970). SAHA and SUMNER (1974) have studied the fate of ^{14}C-lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane), another commonly used pesticide and shown that about 75% of the compound is retained in bread, either as such or as its degradation products, which may be hazardous to human health. Thus, radiation disinfestation offers the additional advantage of leaving no toxic residue.

Decrease in gelatinization viscosity (Fig. 3) may be attributed to amylase-susceptible starch degradation on prolonged storage (D'APPOLONIA *et al.*, 1971). We have observed that glutenin–gliadin ratio in freshly harvested samples increased gradually during four months of storage (from 0.33 to 1.4). The loaf volume is shown to be directly related to the amount of glutenin in the sample (HOSENEY & FINNEY, 1971). Though improvement in baking quality by low dose irradiation of wheat, using lean formula, has been reported (BROWNELL *et al.*, 1955), information on the possible effect of fumigation is incomplete. DEAN and SWANSON (1911) have concluded that the effect of common fumigants on the baking quality of wheat is negligible. Our results are in agreement with this observation. However, repeated fumigation with *Calandrex*, a mixture of trichloroethylene : carbon tetrachloride : carbon bisulfide (64 : 10 : 26, w/w/w), resulted in a marked decrease in loaf volume (CALDERSON *et al.*, 1970). Similarly, excessive dosage of methyl bromide (BURNSBROWN *et al.*, 1961) and carbon tetrachloride (LARMOUR & BERGSTEINSSON, 1938) were found to be detrimental to baking qualities of wheat, causing some discoloration of bread.

There is scant information on the acceptability of breads prepared from irradiated wheat. Bread and *Chapaties* made from irradiated wheat, were highly acceptable organoleptically up to 8 months. These results are in agreement with the report on irradiated (up to 100 krad) and stored *Pakistani wheat* (AHMAD *et al.*, 1973), though MILLER and co-workers (1965) have reported detectable off-flavour in breads, made from irradiated wheat. (at 125 krad).

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RAPID INSTRUMENTAL ANALYSIS OF COMPOSITION OF WINE

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Preliminary studies were made on two types of wines – a white *Sauterne* and a red *Burgundy* – in the near-infrared wave-length region to determine if the infrared absorption technique can be used for rapid determination of the composition of wine.

A computerized spectrophotometer developed in the US DEPARTMENT OF AGRICULTURE, ARS, INSTRUMENTATION RESEARCH LABORATORY (USA) was used for the absorption measurements. Six wave-lengths such as: 1.0720, 1.4504, 1.6960, 1.8160, 2.1504 and 2.2320 μm were found to be the most characteristic for the main components (alcohol, sugar, acid and water) of wine.

Four optical density differences were formed from the optical density (OD) values measured at these wave-lengths:

$$\varphi_1 = OD_{\lambda 1.4504} - OD_{\lambda 1.0720}$$
$$\varphi_2 = OD_{\lambda 1.8160} - OD_{\lambda 1.0720}$$
$$\varphi_3 = OD_{\lambda 1.8160} - OD_{\lambda 1.6960}$$
$$\varphi_4 = OD_{\lambda 2.1504} - OD_{\lambda 2.2320}$$

The relationship between these physical parameters, φ_1, φ_2, φ_3, and φ_4 and the compositional data from chemical analysis were determined for 26 wine samples by regression analyses. The error in predicting the composition by the near-infrared analysis was less than 0.1 vol % for alcohol, 2 g l^{-1} for sugar and 1 g l^{-1} for acid. The composition of the wine samples made artificially from the two original wines ranged from 11.88 to 17.47 vol % alcohol, 8.34 to 88.47 g l^{-1} sugar, and 4.89 to 82.63 g l^{-1} acid. The sample thickness was 1 mm.

With further investigation, a better and quite satisfactory accuracy can be expected for all the main components of the wine by using the optimal sample thickness for each wave-length region, determining the ideal wave-lengths by correlation analysis, working with higher light intensity, utilizing the information hidden in the first and second derivative of the spectral OD curves.

This investigation led to the conclusion that the infrared technique is suitable for a rapid instrumental determination of the composition of wine. It has the advantage that all the main components can be determined and all with the same sensor and instrument using only different interference filters to isolate the desired wave-lengths.

A rapid analysis of composition of wine is becoming increasingly important and application techniques which can be used by unskilled personnel are desired. Therefore, the chemistry involved in separating and purifying components must be eliminated or we must devise automatic instruments to do the operations required for the analysis. Automatic chemical analysis devices represent one solution and much progress is being made in developing such devices; however, instruments which could indicate the composition without the chemical steps would be preferred.

The "measurement alteration" method was developed (KAFFKA, 1967) in the CENTRAL FOOD RESEARCH INSTITUTE in Hungary for rapid determination of the composition of different foodstuffs. A spectrophotometric tech-

nique was developed (BUTLER & NORRIS, 1960; NORRIS & BUTLER, 1961) in the US DEPARTMENT OF AGRICULTURE, ARS, INSTRUMENTATION RESEARCH LABORATORY in the USA for the rapid determination of the composition of different agricultural products. According to the first method, the values of several classical physical parameters of the samples are measured and, using the matrix calculation, the wanted compositional data are transformed from the measured values. In the second method, the optical density (OD) of the sample is measured at different given wave-lengths and the composition is determined using multiple regression analysis.

It seemed to be very useful to compare the two methods and, of course, a higher accuracy and a wider-ranging application was expected by combining them. Each could complement the other.

The investigation of the wine was the first step to compare the two methods. The goal was to work out a method for the rapid, accurate determination of the content of as many different wine components as possible as the basis for developing an instrument for this purpose.

The measurement alteration method was used successfully for determination of composition of liquor (KAFFKA & GÖNCZY, 1969a), of pickling brine (KAFFKA & GÖNCZY, 1969b), of butter (GÖNCZY *et al.*, 1970), and of wine (KAFFKA, 1971). The investigated wine was considered to contain three components: alcohol, soluble extracts, and the entirety of all other constituents. Composition was determined by measuring specific gravity and refractive index.

The spectrophotometric technique was successfully used for determination of the main components of eggs (NORRIS & ROWAN, 1962), of grain (MASSIE & NORRIS, 1965), of plant materials (NORRIS, 1965), of fruits (BITTNER & NORRIS, 1968), of meat products (BEN-GERA & NORRIS, 1968a), and of milk (BEN-GERA & NORRIS, 1968b), but it has not been used for determination of composition of wine.

1. Materials and methods

Two types of wine were investigated: a white *Sauterne* and a red *Burgundy*. Both were made and bottled by the ITALIAN SWISS COLONY IN CALIFORNIA. The temperature of both of the wine samples and the laboratory were kept at 25 °C. Known amounts of alcohol, fructose and tartaric acid were added to the two original wines to obtain 26 samples with different known compositions.

The chemical analyses of the two original wines were made by the DEPARTMENT OF FOOD SCIENCE, NORTH CAROLINA STATE UNIVERSITY at Raleigh, and by the DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY, CORNELL UNIVERSITY, at Geneva, N. Y. Analyses to determine alcohol, sugar and acid content were made in different ways.

The optical density (transmittance) measurements were made with a spectrophotometer constructed in the USDA, ARS, INSTRUMENTATION RESEARCH LABORATORY. This instrument consists of a double monochromator (*Cary* 14) coupled to a digital computer using the single-beam principle previously described (MASSIE & NORRIS, 1971). A large area lead sulfide photocell was used as the detector.

The samples were contained in a metal-walled cell with glass windows where 0.3 mm, 1 mm, and 5 mm sample thicknesses were available. A 2 mm wide slit was chosen which provides a spectral bandpass of less than 8 nm for the wave-lengths used. The transmittance data were recorded on magnetic tape as optical density ($OD = \log I_0 - \log I_s$, where I_0 is the incident light intensity and I_s the light intensity transmitted by the sample) in the 0.8 to 2.4 μm wave-length region by 0.8 nm steps (2001 data were recorded in the whole wave-length region). The sensitivity of the measurement of the optical density – the vert cal sensitivity of the recorded spectral curve – was 0.0003 OD/step (1 OD corresponds to 3.276 steps).

The mathematical formulas of the measurement alteration method (KAFFKA, 1967) and those of the multiple regression analysis method were equally used to evaluate the data measured with instruments on the one hand and determined by chemical analysis on the other.

2. Results

The spectral OD curves of the *Sauterne* wine are shown in Figs. 1, 2 and 3. The OD curves were obtained through a cell with 1 mm sample thickness. We can see the effect of adding 6 vol% alcohol (Fig. 1), 8 weight% sugar (Fig. 2), and 8 weight% tartaric acid (Fig. 3). For comparison, the OD curves of distilled water and the wine samples are shown in detail (magnified 4×) in the middle of the wave-length region. The spectral OD curves of the *Burgundy* wine with added alcohol, sugar and tartaric acid – similar to the previous ones — are shown in Figs. 4, 5 and 6. In the next three figures, we can see the spectral OD curves of the three main components of the wine: the absolute pure ethyl alcohol (Fig. 7), the saturated sugars, fructose and dextrose (Fig. 8), and the solution containing 4 weight% tartaric acid (Fig. 9). In Fig. 10 we can see the spectral OD curves of the *Sauterne* and *Burgundy* wines obtained through a cell with 5 mm sample thickness. The ordinate for *Burgundy* is shifted up 1 OD in order to better separate the curves. In the shorter wave-length region, we can see the curves magnified 4 times as well. The data for all these curves are not valid above 3 OD because of straylight limitations of the monochromator.

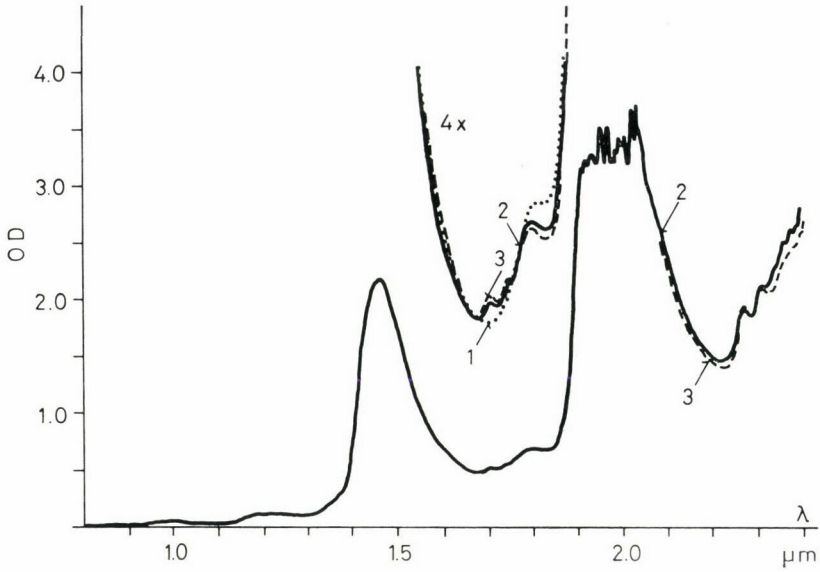

Fig. 1. The spectral OD curve of *Sauterne* wine and the effect of adding 6 vol % alcohol. Sample thickness 1 mm. Width of slit 2 mm. 1: Dist. water; 2: *Sauterne* wine; 3: *Sauterne* wine + 6 vol % alcohol

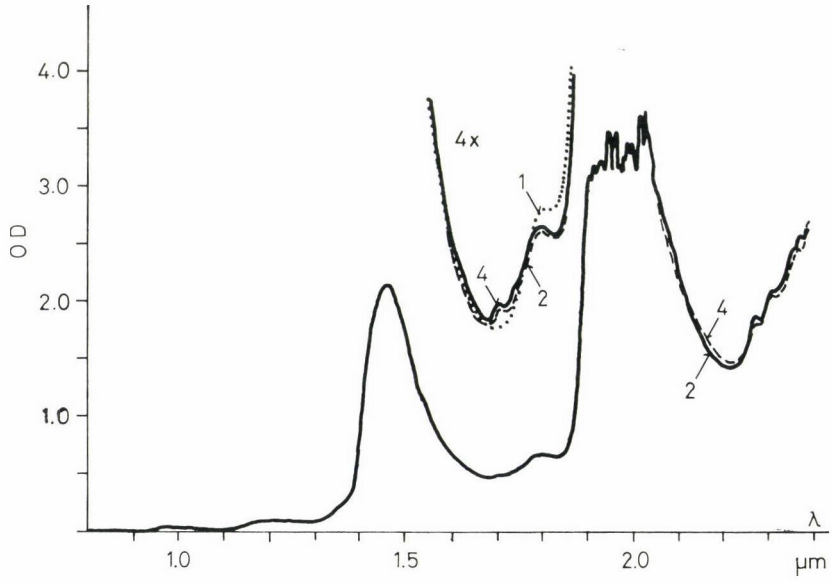

Fig. 2. The spectral OD curve of *Sauterne* wine and the effect of adding 8 weight % sugar. Sample thickness 1 mm. Width of the slit 2 mm. 1: Dist. water; 2: *Sauterne* wine; 4: *Sauterne* wine + 8 w % sugar

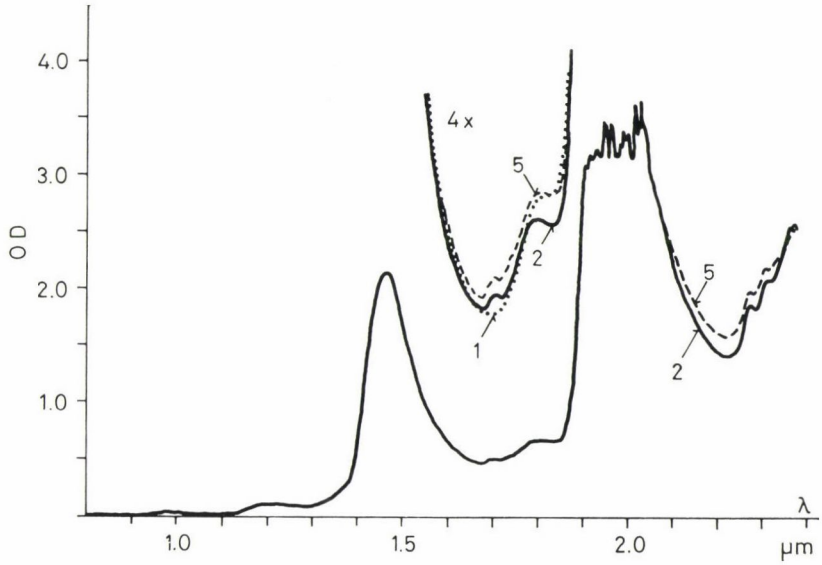

Fig. 3. The spectral OD curve of *Sauterne* wine and the effect of adding 8 weight % acid. Sample thickness 1 mm. Width of the slit 2 mm. 1: Dist. water; 2: *Sauterne* wine; 5: *Sauterne* wine + 8 w % tartaric acid

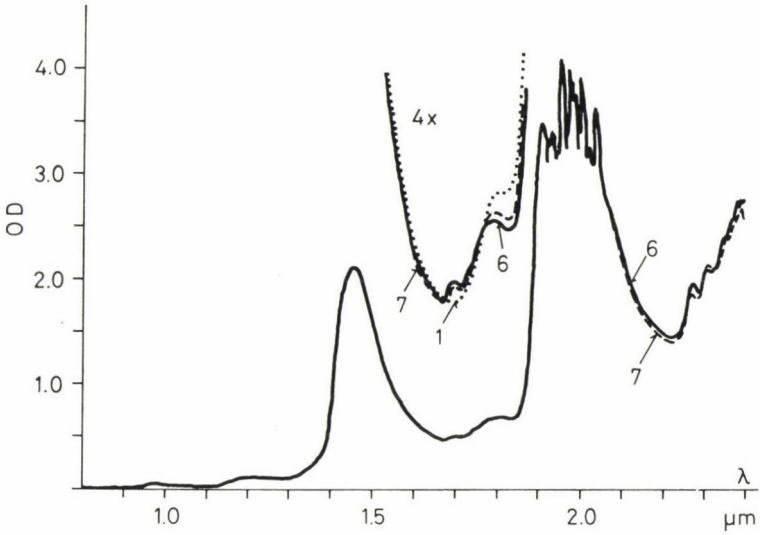

Fig. 4. The spectral OD curve of *Burgundy* wine and the effect of adding 6 vol % alcohol. Sample thickness 1 mm. Width of the slit 2 mm. 1: Dist. water; 6: *Burgundy* wine; 7: *Burgundy* wine + 6 vol % alcohol

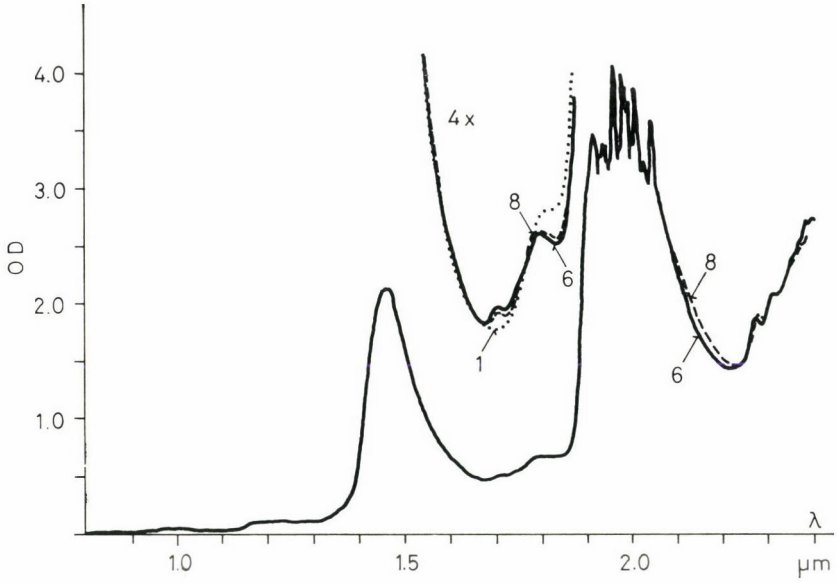

Fig. 5. The spectral OD curve of *Burgundy* wine and the effect of adding 8 weight % sugar. Sample thickness 1 mm. Width of the slit 2 mm. 1: Dist. water; 6: *Burgundy* wine; 8: *Burgundy* wine + 8 w % sugar

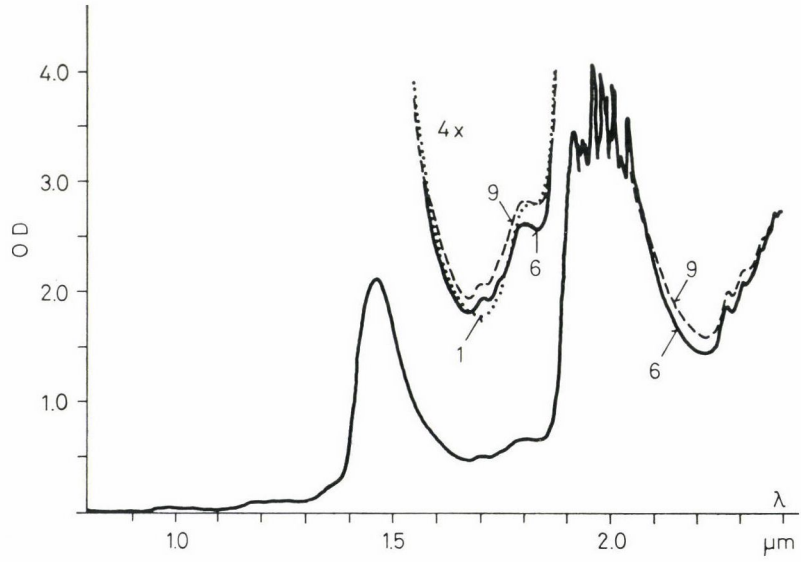

Fig. 6. The spectral OD curve of *Burgundy* wine and the effect of adding 8 weight % acid. Sample thickenss 1 mm. Width of the slit 2 mm. 1: Dist. water; 6: *Burgundy* wine; 9: *Burgundy* wine + 8 w % tartaric acid

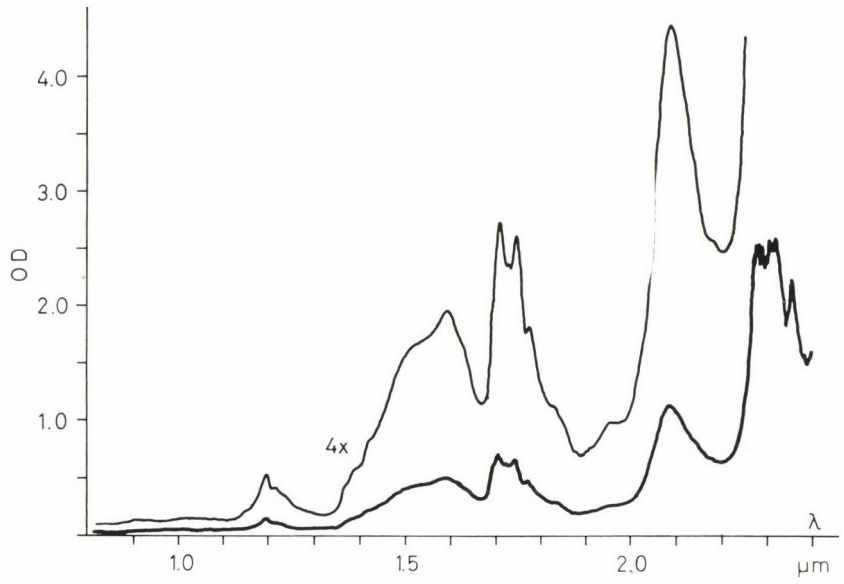

Fig. 7. The spectral OD curve of absolute pure ethyl alcohol. Sample thickness 1 mm. Width of the slit 2 mm

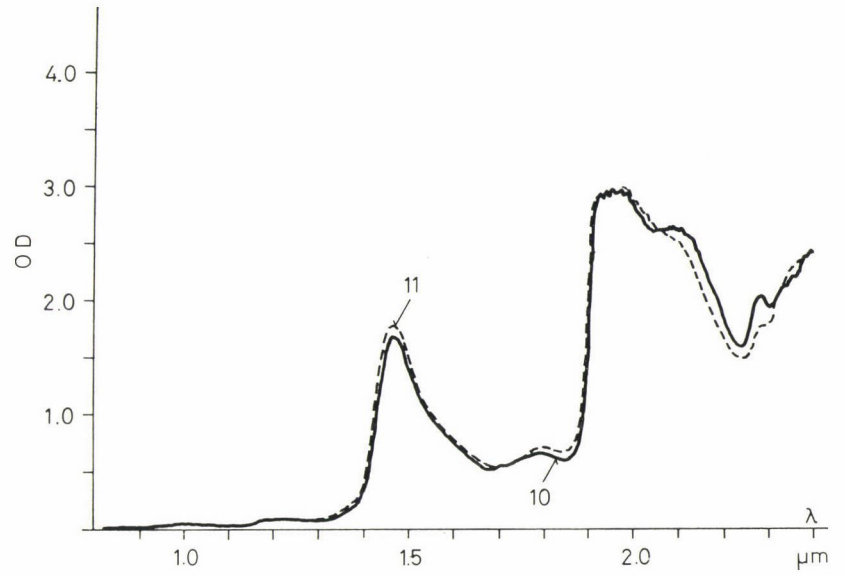

Fig. 8. The spectral OD curves of saturated aqueous solutions of fructose and dextrose. Sample thickness 1 mm. Width of the slit 2 mm. 10: Fructose; 11: dextrose

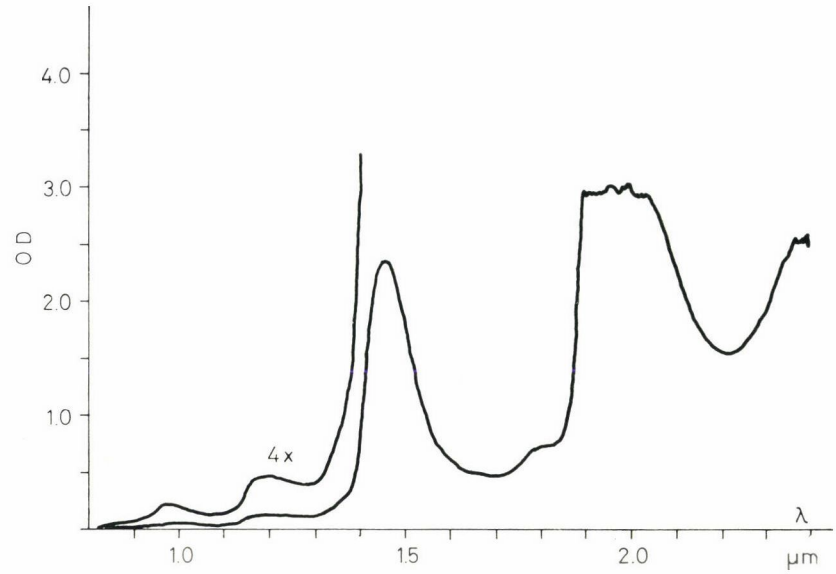

Fig. 9. The spectral OD curve of aqueous solution containing 4 weight % tartaric acid. Sample thickness 1 mm. Width of the slit 2 mm

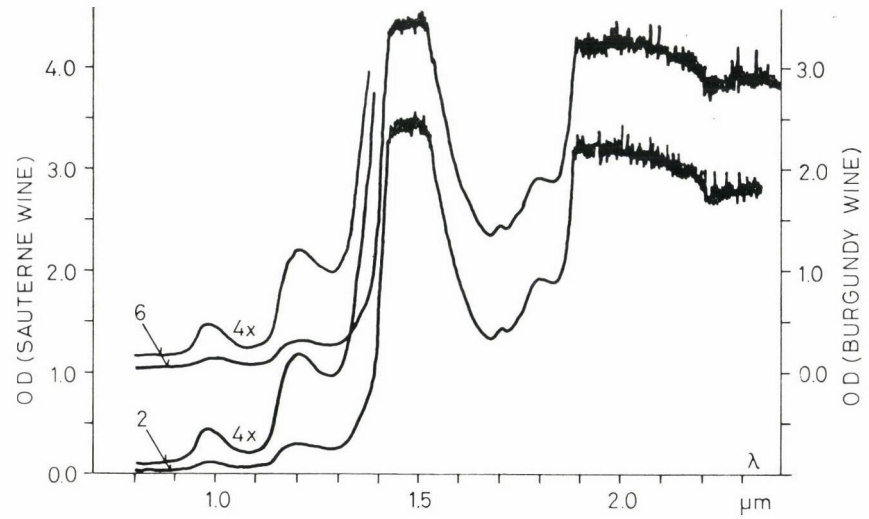

Fig. 10. The spectral OD curves of *Burgundy* and *Sauterne* wines. Sample thickness 5 mm. Width of the slit 2 mm. 2: *Sauterne* wine; 6: *Burgundy* wine

We chose 12 wave-lengths which we found characteristic from the shape of the spectral OD curves:

$\lambda_0 = 1.0720$ μm
$\lambda_1 = 1.1600$ μm
$\lambda_2 = 1.1864$ μm
$\lambda_3 = 1.2664$ μm
$\lambda_4 = 1.4504$ μm
$\lambda_5 = 1.6600$ μm
$\lambda_6 = 1.6960$ μm
$\lambda_7 = 1.8160$ μm
$\lambda_8 = 2.1504$ μm
$\lambda_9 = 2.2168$ μm
$\lambda_{10} = 2.2320$ μm
$\lambda_{11} = 2.2688$ μm

We found the data at λ_0, λ_3, λ_5, and λ_9 to be good reference values for eliminating the zero level shift. The data at λ_1, λ_2, λ_6, λ_7, and λ_{11} were characteristic for alcohol, the spectral OD curve having sharp local maxima (peaks) at λ_2, λ_6, and λ_{11} and local maxima at λ_1 and λ_7. The spectrum of water has a sharp peak at λ_4. The spectrum of tartaric acid has two broad maxima but λ_7 seemed to be the most characteristic. Sugar has one small broad maximum at λ_8.

We also investigated the fluctuation of the data along the spectral OD curve of the wine. We found that using 1 mm sample thickness at λ_0, λ_1, λ_2, λ_3, λ_5, λ_6, and λ_7 this fluctuation does not exceed the vertical sensitivity; that means it does not exceed the 0.0003 OD value, while the fluctuation of the data is 0.005 OD at λ_4 and 0.010 OD at λ_8, λ_9, λ_{10}, and λ_{11}. The effect of this fluctuation, in other words, the effect of the noise of the spectral curve can be reduced by averaging several data points.

Table 1

The values of the physical parameters – the Δ OD values, for 1 mm thickness, at the given wave-lengths – and the compositional data of the 13 Sauterne wine samples

Sample No.	φ_1	φ_2	φ_3	φ_4	Sugar g l^{-1}	Acid g l^{-1}	Alcohol vol %
1	2.1543	0.6425	0.1593	0.2112	8.80	5.16	12.42
2	2.1292	0.6385	0.1498	0.2057	8.68	5.09	13.74
3	2.1111	0.6326	0.1407	0.2016	8.57	5.02	15.01
4	2.0865	0.6265	0.1312	0.1984	8.46	4.96	16.26
5	2.0623	0.6208	0.1221	0.1939	8.34	4.89	17.47
6	2.1362	0.6422	0.1556	0.2265	28.50	5.11	12.29
7	2.1254	0.6420	0.1521	0.2421	47.80	5.05	12.17
8	2.1097	0.6411	0.1486	0.2587	66.57	4.99	12.02
9	2.0933	0.6391	0.1451	0.2683	84.93	4.93	11.88
10	2.1334	0.6620	0.1643	0.2090	8.73	24.96	12.32
11	2.1169	0.6789	0.1692	21.63	8.64	44.34	12.19
12	2.0949	0.6885	0.1724	0.2150	8.55	63.30	12.06
13	2.0745	0.7053	0.1782	0.2182	8.47	81.92	11.95

After having analyzed the data at the given 12 wave-lengths, we found the following differences most characteristic:

$$\varphi_1 = OD_{\lambda 1.4504} - OD_{\lambda 1.0720}$$
$$\varphi_2 = OD_{\lambda 1.8160} - OD_{\lambda 1.0720}$$
$$\varphi_3 = OD_{\lambda 1.8160} - OD_{\lambda 1.6960}$$
$$\varphi_4 = OD_{\lambda 2.1504} - OD_{\lambda 2.2320}$$

For these parameters φ_1, φ_2, φ_3, and φ_4, average values were calculated from seven data; namely, from the OD value at the given wave-length and from the three OD values on either side of this wave-length, representing 7 data in a band-width of 5.6 nm. We also calculated data with an average from 15 and 27 data, resp., with a 12.0 nm and 21.6 nm band-width but these were unsatisfactory because sharp peaks were masked. The data obtained through a cell with 1 mm sample thickness are shown in Table 1 for the 13 samples of *Sauterne* wine and in Table 2 for the 13 samples of *Burgundy* wine, the two tables contain the compositional data of the same samples as well.

To determine the equation system describing the relationship between the compositional data and physical parameters, we used first the "STEPR" stepwise multiple regression program developed by P. D. SCHOCH and modified by S. H. CROSSLAND at GODDARD COMPUTER SCIENCE INSTITUTE. Using the measurement alteration method, we realized that after several steps it leads

Table 2

The values of the physical parameters – the Δ OD values, for 1 mm thickness, at the given wave-lengths – and the compositional data of the 13 Burgundy wine samples

Sample No.	φ_1	φ_2	φ_3	φ_4	Sugar g l^{-1}	Acid g l^{-1}	Alcohol vol %
14	2.1489	0.6421	0.1623	0.2033	12.50	5.89	12.42
15	2.1273	0.6370	0.1525	0.2034	12.34	5.81	13.74
16	2.1056	0.6312	0.1446	0.1996	12.16	5.73	15.00
17	2.0828	0.6255	0.1350	0.1984	12.00	5.65	16.25
18	2.0611	0.6207	0.1256	19.68	11.85	5.58	17.46
19	2.1351	0.6418	0.1589	0.2154	32.16	5.83	12.29
20	2.1212	0.6412	0.1556	0.2327	51.42	5.77	12.16
21	2.1058	0.6411	0.1525	0.2472	70.15	5.70	12.02
22	2.0921	0.6391	0.1489	0.2637	88.47	5.63	11.88
23	2.1279	0.6580	0.1668	0.2033	00.124	25.68	12.32
24	2.1115	0.6727	0.1721	0.2052	12.27	45.06	12.19
25	2.0918	0.6889	0.1772	0.2034	12.14	64.01	12.06
26	2.0741	0.7035	0.1818	0.2071	12.02	82.63	11.95

to the same partial results as the "STEPR" program so the same end-result is to be expected. The calculation was actually done by a *Data General Corporation Computer*, type *Nova* 1200.

We found the following equation systems for *Burgundy* and *Sauterne* wines:

Burgundy

$$\text{Alcohol (vol\%)} = -18.38\ \varphi_1 - 87.7\ \varphi_3 - 45.8\ \varphi_4 + 75.44$$

$$\text{Sugar (g l}^{-1}) = -190.7\ \varphi_3 + 1236.3\ \varphi_4 - 207.4$$

$$\text{Acid (g l}^{-1}) = -314.8\ \varphi_1 + 770.1\ \varphi_2 + 337.9\ \varphi_3 - 196.2\ \varphi_4 + 172.9$$

Sauterne

$$\text{Alcohol (vol\%)} = 39.18\ \varphi_2 - 142.1\ \varphi_3 - 39.4\ \varphi_4 + 18.14$$

$$\text{Sugar (g l}^{-1}) = -126.35\ \varphi_1 - 297.3\ \varphi_2 + 1108.4\ \varphi_4 + 239.3$$

$$\text{Acid (g l}^{-1}) = -436.9\ \varphi_1 + 450.4\ \varphi_2 + 876\ \varphi_3 - 193.5\ \varphi_4 + 557.2$$

We found the following standard errors:

for *Burgundy*		for *Sauterne*	
σ_{alcohol}	= 0.07 vol%	σ_{alcohol}	= 0.09 vol%
σ_{sugar}	= 1.83 g l^{-1}	σ_{sugar}	= 2.01 g l^{-1}
σ_{acid}	= 0.69 g l^{-1}	σ_{acid}	= 1.24 g l^{-1}

For comparison we determined the equation system describing the relationship between the compositional data and the two previously used classical physical parameters (KAFFKA, 1971) such as specific gravity and refractive index of the same wine samples, and the standard errors as well. For this simple equation system we found the following standard errors:

for *Burgundy*		for *Sauterne*	
σ_{alcohol}	= 0.86 vol%	σ_{alcohol}	= 0.85 vol%
σ_{extracts}	= 1.26 g l^{-1}	σ_{extracts}	= 0.63 g l^{-1}

This method is not usable for more components of the wine because it measures only two physical parameters.

3. Conclusions

Although the described investigation of wine in the near-infrared can be taken only as a preliminary study, partly because we investigated only two types of wine making the 26 samples by artificially adding alcohol, sugar and acid to them, it led to the conclusion that the infrared technique developed in the USDA, ARS, INSTRUMENTATION RESEARCH LABORATORY is very suitable for a rapid instrumental determination of the composition of wine.

The investigation showed that the studied wave-length region could be divided into three parts by the two main water peaks at about 1.45 μm and 1.94 μm. In the first (shorter wave-length), the pigments in red wines have some small influence on the spectra, but only below 1.05 μm. In this wave-length region, a 5–25-mm sample thickness seemed to be the most suitable for the

measurement. In the second region – from 1.45 to 1.94 μm – a 1–5-mm sample thickness seemed to be the best one; in the third region – above 1.94 μm – 0.2–1 mm.

We used only the data obtained through a 1-mm sample thickness for determination of the equation system. Under these circumstances, we obtained very good accuracy for alcohol determination. It is better than that obtained by the measurement alteration method using only classical physical parameters and it is about the same accuracy as the best chemical analysis. The accuracy for acids is just acceptable while the accuracy for sugar is not quite satisfactory. Probably using a more suitable sample thickness for sugar determination, perhaps modifying the chosen wave-lengths and computing the average from more data (from a broader wave-length region), and working with a higher light intensity, we will get the required accuracy for this component as well. It may help to use a classical physical parameter (such as the refractive index or the specific gravity) as an auxiliary parameter. This requires, however, further and deeper investigation.

We found also that the first and second derivatives of the spectral OD curves contain additional information which can be used to improve the accuracy in predicting composition of wine.

The infrared technique has the advantage that the same instrument can be used at different wave-lengths – for many components – changing only the interference filters in it, while measuring each classical physical parameter requires a different device.

*

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RECENT DATA ON THE STRUCTURE OF PANGAMIC ACID (VITAMIN B_{15})

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The biological activity and pharmacological behaviour of natural pangamic acid (vitamin B_{15}) as characterized by lipotropic and detoxification effects, participation in the enhanced oxygen exchange of cells, clearly show the important role of this compound in the intermediary metabolism. Synthetic pangamic acid samples, however, differ in their characteristics, and therefore it seemed necessary to study the structure and the chemical behaviour of synthetic preparations. First IR spectra, elemental composition, then conductivity of a certain preparation have been investigated and it was found that its structure undergoes some marked changes as a function of pH value of the solution: and a protonized quaternary N atom is formed. On the basis of such a structure the analytical difficulties met at the investigation of pangamic acid samples of various origin, on one hand, and their lipotropic behaviour on the other, could be easier interpreted.

As a result of biochemical research in the past decades, a growing number of biologically active substances has been discovered. Pangamic acid or vitamin B_{15}, a member of the vitamin B complex, became known only in the past 20 years. Although the function of vitamin B_{15} in the living organism has not yet been fully elucidated, its physiological activity and pharmacodynamical properties indicate that pangamic acid is a very effective biocatalyst occurring both in plants and animals.

Krebs and co-workers (1951) were the first to isolate the compound from the aqueous extract of apricot stones and named it pangamic acid. The name was derived from the fact that the compound is found in high concentrations mainly in seeds.

In 1953 Tomiyama and Yone isolated a similar substance from the aqueous extract of bull's liver, where it occurred in the same fraction as vitamin B_{12}, folic acid and other members of the vitamin B group. He, therefore, named it vitamin B_{15}.

Krebs and Johnson (1955) established and confirmed the identity of the two compounds. Since then, both names are used in the literature. These scientists also reported on the synthesis of pangamic acid and its derivative (Krebs & Krebs, 1955).

Bukin and Garkina (1965) prepared the Ca salt of pangamic acid by a path differing from the Krebs synthesis. Subsequently Yurkevich and coworkers (1965) proposed further methods for synthetizing vitamin B_{15} and its analogues. They published the physical and chemical properties of the basal substance prepared and the structure assumed by them, namely D-glu-

cono-dimethylaminoacetate, *i.e.* the dimethylglycine ester of D-gluconic acid (Fig. 1). This is a white, crystalline, very hygroscopic substance. Like all members of the vitamin B group, it is readily soluble in water, and less soluble in solvents having high dipole moments. In fat solvents it is not soluble at all.

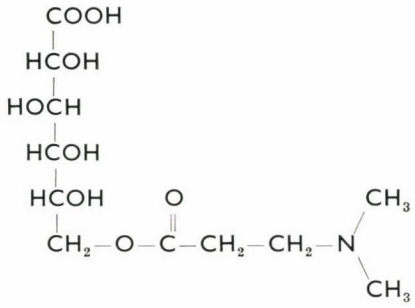

Fig. 1. Pangamic acid (vitamin B_{15})

The physiological role of pangamic acid can be summarized as follows:

— *It is one of the lipotropic factors.* The fat content in the liver of animals kept on a protein-free diet decreases when pangamic acid is administered (SOKOLOVA, 1965); pangamic acid increases the creatine phosphate content in the muscles and the activity of the transketolase enzyme of the liver (YAKOVLEV *et al.*, 1965); it participates also as methyl donor in creatine synthesis (GARKINA & SOLOVYEVA, 1965).

— *It participates in the active oxygen exchange of cells and tissues.* It increases the activity of succinic dehydrogenase in the mitochondria of the liver (SHAMSOI & SELESNEVA, 1969), as well as the intensity of endogenous respiration and the effect of glucose on oxygen consumption (KECHKUASVILI & KOMETIANI, 1969). As a consequence of continued pangamic acid administration the activity of cytochrome-oxidase is also increased (SAMODONOVA, 1969).

— *It has a detoxicating effect.* It suppresses the toxic effect of carbon tetrachloride and chloroform (AZHGIKIN & YAKUPOVA, 1969); it has successfully been applied in the chemotherapy of experimental tumors (POLYAK, 1970).

In recent years, extensive research on pangamic acid is being carried out, directed towards the elucidation of its physiological role and of the possibilities of its therapeutical applications. In most cases clinical and pharmacodynamical investigations utilized synthetic pangamic acid preparations. However, the structure and composition of these preparations do not usually correspond to the formula described by KREBS. It therefore appears necessary for the elucidation of the exact biological role of pangamic acid, to determine the real structure, composition and chemical properties of pangamic acid

isolated from natural substances. Only a few research teams are concerned with the pangamic acid content of natural substances, with its isolation and with the structural and chemical problems of the isolated compound.

1. Materials and methods

Pangamic acid (vitamin B_{15}) manufactured by NUTRITIONAL CORPORATION, Cleveland, Ohio, was used in our investigations. This product was chosen because from the product characteristics listed by the manufacturing company, it appeared that its composition and structure corresponded closely to the KREBS formula.

1.1 IR spectra

IR spectra were taken in KBr tablets, using a UR-20 *Infrared Spectrometer*.

1.2. Adjusting of pH values

A 10 mg ml^{-1} solution of pangamic acid was prepared, and pH was set with 1 *N* HCl to values of 2, 3, 4 and 5, resp. The pH value of the aqueous stock solution was 5.5–5.6. pH values were measured with a *Radelkisz* OK 102 pH-meter.

1.3. Thin-layer chromatography

For thin-layer chromatographic determination of pangamic acid, the method developed at the DEPARTMENT OF BIOCHEMISTRY AND FOOD TECHNOLOGY, TECHNICAL UNIVERSITY OF BUDAPEST was used (TELEGDY KOVÁTS *et al.*, 1970).

1.4. Conductivity measurements

A *Radelkisz* OK 102/1 conductometer was used for these measurements. First the changes in conductivity of pangamic acid solutions *vs.* pH, were measured by successively adding 1 *N* HCl to the solution and checking the pH values. In a second series of measurements 1 *N* HCl was added in exactly the same amounts as earlier, to distilled water, to determine pH changes in the solution containing no pangamic acid. The conductivity of pangamic acid was then calculated from the difference between the two curves.

2. Results

It may be seen from the formula that pangamic acid contains two methyl groups bound to N. It seems probable that the lipotropic effect of pangamic acid is due to labile methyl groups. A comparison of this structure, however, with

that of other biological methylating agents, shows that activation – sulphonium groups, quaternary N atoms – characteristic of labile methyl groups can not be detected. Therefore, now the structure of individual synthetic pangamic acid preparations is studied as well as the structural changes effected by various chemical influences *e.g.* by pH variations.

It is well known that the structure of the various synthetic pangamic acid preparations does not always agree with the basal structure described by KREBS. The synthetic preparations frequently contain other components, too: gluconic acid, dimethylglycine, even the tetra- and octamethyl derivatives of the basal compound. It is therefore necessary to know the structure and composition of the pangamic acid preparation used as standard, to be able to compare it with the pangamic acid isolated from natural substances.

Another question deserving attention is the change of the stability of the ester bond of pangamic acid, with pH. The isolation of pangamic acid from natural substances is usually performed at strongly acid pH values, and hence it is highly important to know which pH values do not cause hydrolysis of the ester bond, allowing at the same time the removal of the interfering substances.

The elemental analysis of the preparation gave results (Table 1) which indicate that the composition of the investigated product is in good agreement with the composition calculated from the assumed formula of pangamic acid. The difference between found and calculated values is presumably due to traces of water in the sample.

Table 1

Data of elementary analyses

Sample	Calculated %	Measured %
Nitrogen	5.0	5.22
Carbon	42.7	39.9
Hydrogen	6.76	7.27

The IR absorption spectrum of the preparation clearly shows the double carbonyl band at 1650–1740 cm^{-1} characteristic of the structure of pangamic acid (Fig. 2). The spectrum is in good agreement with IR spectra of pangamic acid published in the literature. Thus, preliminary studies confirm that the pangamic acid preparation used in the investigations consists of the dimethylglycine ester of D-gluconic acid.

Then the change of pangamic acid structure *vs.* pH was studied. These studies were carried out only in the acid pH range, since – according to data in the literature – in the neutral and alkaline range the ester bond is easily decomposed.

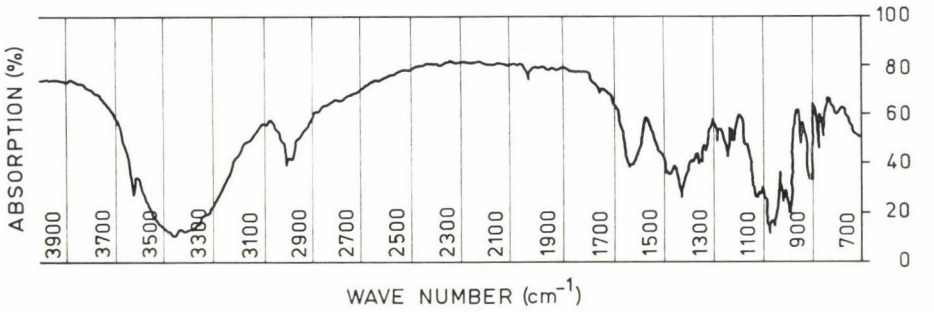

Fig. 2. Infrared absorption spectrum of pangamic acid

The thin-layer chromatographic behaviour of pangamic acid solutions in the 2.0–5.5 pH range has also been studied. It was assumed that, in case the ester bond had been hydrolysed at a certain pH value, a new spot appears on the chromatogram beside the original one. The results, however, indicated that no hydrolysis of the ester bond took place in the solutions: the R_f values of the spots were identical at all pH values.

The IR absorption spectra of pangamic acid samples obtained by crystallization from the solutions with different pH values have shown an intense change in the wave number ranges 1600–1800 cm^{-1} and 2300–2500 cm^{-1}, resp. (Fig. 3).

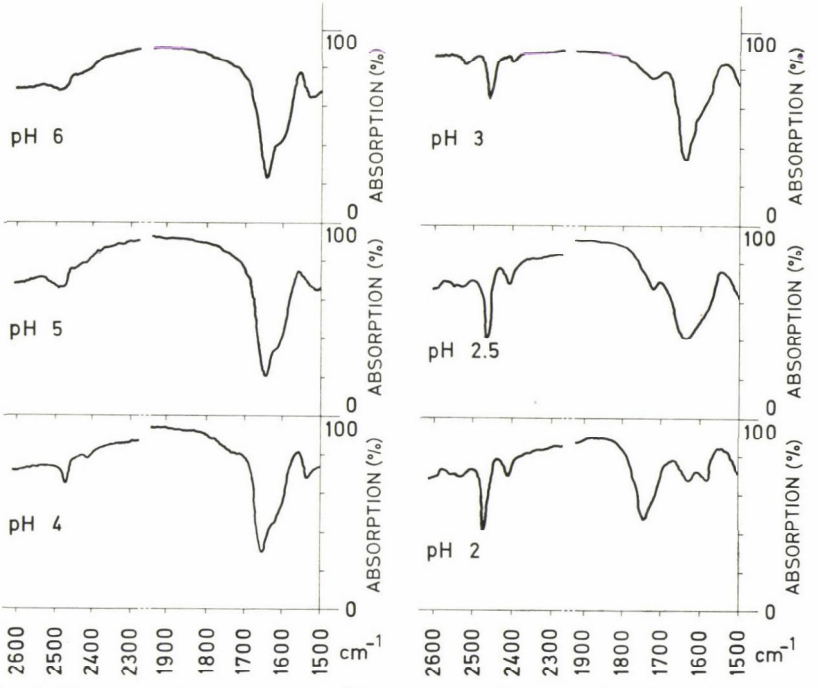

Fig. 3. Changes of IR spectra of pangamic acid as a function of pH values

It may be seen from the figure that, with decreasing pH values, the absorption band at 1650 cm^{-1}, characteristic of the carbonyl group, substantially weakens. At pH 3 a new band appears at 1730 cm^{-1}. At pH 2, the intensity of this new band already exceeds that of the former band. The wave number of the carbonyl band at 1650 cm^{-1} is very low.

A significant change can also be observed in the 2300–2500 cm^{-1} wave number range. With decreasing pH values, a medium-strength band appears at 2420 cm^{-1} and a strong band at 2480 cm^{-1}. The appearance of these bands unequivocally demonstrates salt formation of the tertiary amine, *i.e.* the presence of the quaternary nitrogen atom.

3. Conclusions

Thus, IR spectra show a structural change of pangamic acid with decreasing pH. This change presumably consists in the protonization of the nitrogen atom and in an increase of the apparent stability of the ester bond.

It was tried to confirm the protonization of the nitrogen atom in another way, too, by studying the change in the conductivity of pangamic acid solutions with decreasing pH (Fig. 4).

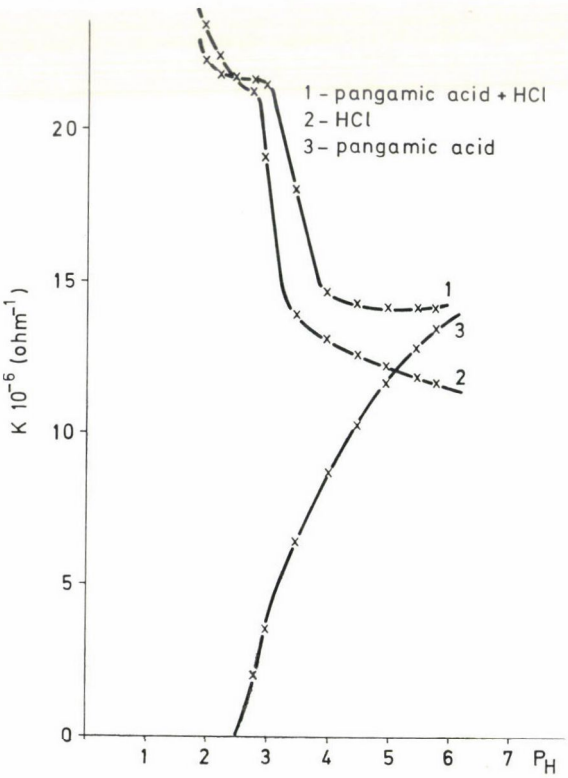

Fig. 4. Changes of conductivity of pangamic acid as a function of the pH value

It may be seen that the conductivity of pangamic acid rapidly decreases with decreasing pH, and becomes zero at pH 2.5, while the conductivity of the hydrochloric acid solution suddenly increases at pH 2.5. These results indicate that up to pH 2.5, the hydrogen ions introduced with hydrochloric acid are bound by pangamic acid, *i.e.* protonization takes places. At pH 2.5, protonization becomes complete, and free hydrogen ions appear which increase conductivity.

Summing up, it may be stated that a structural change takes place in the pangamic acid molecule with decreasing pH which manifests itself above all in the protonization of the tertiary nitrogen atom (Fig. 5).

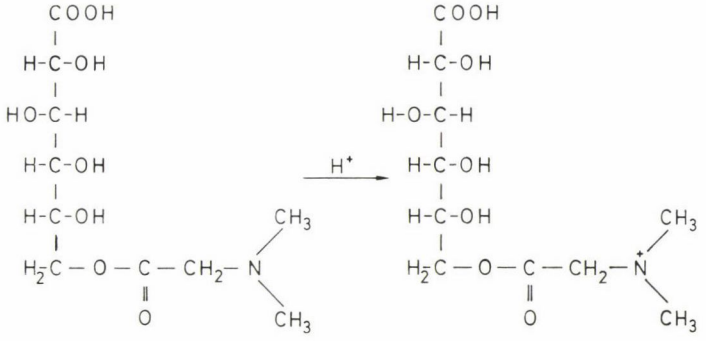

Fig. 5. Assumed mechanism of pangamic acid protonization

IR spectra indicate, however, that the structural change is not confined only to protonization. It may be concluded from the spectra that with decreasing pH, the stability of the ester bond in pangamic acid apparently increases, and the intensity of the ester band also increases. This result is rather surprising, since all earlier experience showed that the stability of ester bonds to hydrolysis is reduced towards extreme pH values.

It must, therefore, be assumed that in pangamic acid under such circumstances some other structural changes also take place.

As a consequence of these changes, the methyl group or groups, eminently important in the compound's physiological activity, become labile by protonization. It seems also probable that under natural conditions pangamic acids of different structures are present in the living organism, and are in dynamic equilibrium with each other. Presumably, the difficulties experienced in the determination of pangamic acid, can be attributed to this phenomenon.

Further studies to elucidate the problems of protonization and other structural changes as well as pangamic acid forms occurring in nature, are under way.

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THE EFFECT OF SODIUM CHLORIDE, GAMMA IRRADIATION AND/OR HEATING ON GERMINATION AND DEVELOPMENT OF SPORES OF *BACILLUS CEREUS* T IN SINGLE GERMINANTS AND COMPLEX MEDIA

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Germination of heat activated spores of *Bacillus cereus* T was more sensitive to sodium chloride in single germinants (1 m*M* L-alanine, inosine, adenosine or guanosine) than in combinations of L-alanine and nucleosides or digest broth. 100 krad, which reduced spore viability by less than 40%, stimulated germination in 0.1 m*M* L-alanine, but not in 20 m*M* L-alanine or digest broth. After 1 Mrad spores germinated only slightly slower than unirradiated spores in salt-free media and were less sensitive to salt. Spores which had been heated or heated and irradiated, germinated very slowly and practically independent of salt concentration (0–9 % w/v).

Spores surviving irradiation and/or heating were more sensitive to salt when judged by colony formation. The effect of heat + radiation was additive and that of radiation + heat synergistic.

The high resistance of bacterial spores to physical and chemical agents and the biochemistry of their germination remains one of the major mysteries of microbiology, and has both theoretical and applied implications. A full understanding of the control of spore germination and outgrowth is important to the safety of non-sterilized food and might allow the reduction of food processes thereby effecting improvements in quality and nutritional value. Despite the common use of sodium chloride as a preservative its effects on germination processes are poorly documented. Combinations of heat and gamma radiation have been shown to act synergistically with respect to spore recovery, but the additional effects of salt have not been investigated. *Bacillus cereus* T was chosen as a model because of the extensive biochemical literature on its germination and outgrowth.

The effect of sodium chloride on germination of spores of *B. cereus* T has been studied in single germinants and a complex medium, and the effects of heat, radiation, and combinations of heat and radiation on germination and colony formation examined.

1. Materials and methods

A single stock spore suspension of *B. cereus* T was produced at 30 °C in aerated "G" medium (STEWART & HALVORSON, 1953). The spores were harvested and washed four times in cold sterile water by centrifugation. The washed spores were suspended in 20 m*M* phosphate buffer pH 8 (ionic strength

0.08) and heat activated at 70 °C for 30 min prior to use (GOULD *et al.*, 1968). Spores were irradiated in a 4 kCi (148 TBq) ^{60}Co source at 820 krad h^{-1} (8.2 kGy h^{-1}) at 24–28 °C without aeration.

Germination was studied in 20 m*M* phosphate buffer (pH 8.0) containing the following known germinants (all 1 m*M*) : L-alanine, inosine, adenosine, guanosine, alanine + inosine, alanine + adenosine, alanine + guanosine. *Hartley*'s digest broth (CRUICKSHANK, 1965) diluted five-fold with phosphate buffer (pH 8.0) was also used as germination medium. The salt sensitivity of germination was studied by adding various concentrations of NaCl ("Analar") to these media. Solutions of germinants were sterilized by membrane filtration (*Millipore*, 0.22 μm) and all concentrations were expressed as m*M* after the addition of spores (initial cell counts $5–10 \cdot 10^7$ ml). The germination media inoculated with spores were incubated in tubes in an aluminium block controlled thermostatically at 30 $\pm$ 0.1 °C, and the change in optical density (OD) of the suspensions followed using a *Biochem* absorptiometer (HILGER & WATTS, London) using a 580 nm peak transmission filter. The OD of the germination mixture was plotted as a percentage of the initial value against incubation duration, and the maximum germination rate (% decrease in OD min^{-1}) estimated from the steepest portion of the curve.

The cell-state distribution was determined by mixing 1 ml of germinating spore suspension with 0.05 ml 4% mercuric chloride to arrest germination and enzymatic changes (FARKAS *et al.*, 1966) and determining the proportions of refractile and germinated spores by phase-contrast microscopy.

The number of viable cells was determined by spreading in duplicate 0.02 ml drops from decimal dilutions in 0.1% peptone water on yeast-glucose agar (YGA) (GOULD *et al.*, 1968) calculated by the method of FARMILOE and co-workers (1954), and normally incubating at 30 °C for 2–4 days, but prolonging incubation when NaCl was added to YGA. The number of heat resistant cells was determined from samples which had been heated at 70 °C for 30 min before plating. Samples of germination media containing salt were diluted $\times 100$ before heat treatment to eliminate possible effect of NaCl on heat resistance and colony formation. The salt sensitivity of colony formation was investigated by adding various concentrations of NaCl to YGA, the water activity (a_w) of these media being estimated as described by BAIRD-PARKER and FREAME (1967)

2. Results and discussion

2.1. The effects of NaCl on development of B. cereus spores in simple systems and complex media

Inhibition of germination by NaCl was greatest in single germinants, less in combined germinants, and least in the complex medium (Fig. 1). The

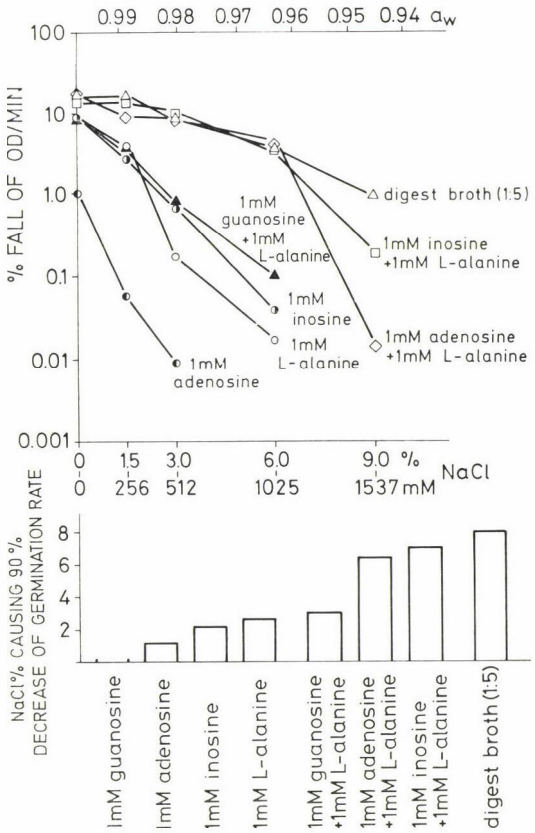

Fig. 1. The effect of NaCl concentration on the germination rate of spores of *B. cereus* T in different germination media. Bottom: the amount of added NaCl causing 90% decrease of germination rate in different germination media

rate of initiation of germination in inosine equalled that in alanine, but that in adenosine was tenfold less in NaCl-free medium and a hundredfold less in the presence of 3% NaCl. Guanosine, practically ineffective in itself as a germinant, increased substantially the NaCl-tolerance of L-alanine-initiated germination, though less than adenosine or inosine.

An immediate reduction of absorbancy of spore suspensions was observed upon addition of NaCl and, in high salt concentrations, spores appeared less refractive than in salt-free medium. Since both these effects may be ascribed to the higher refractive index of salt solution (KAUFMAN, 1960) rather than any alteration in the spores, any immediate optical change was ignored when calculating the germination rate, only the additional fall in OD during incubation being considered.

Differential counts of refractile and germinated spores at intervals during germination (*i.e.* darkening when viewed by phase contrast microscopy)

showed that it was progressively slowed by increasing concentration of NaCl, and also that the swelling of germinated spores was inhibited by as little as 1.5% NaCl.

The different salt sensitivities of the various germinating systems tested support the conclusion (WARREN, 1968) that several independent but parallel chains of reaction may occur in biochemical mechanisms of germination of *B. cereus* T spores, a concept further supported by several germination systems having different time-temperature requirements for activation of the optimal response (SOGIN *et al.*, 1972).

Enriched media are commonly recommended for enhancing the recovery of heat treated spores (SUPPLE & DENGLER, 1916; FRANK & CAMPBELL, 1955; AMAHA & SAKAGUCHI, 1957; EDWARDS *et al.*, 1965a, b; CAMPBELL *et al.*, 1965; AUGUSTIN & PFLUG, 1967) and there are some indications that the requirements for recovery of heat damaged spores are specifically for initiation of germination and not for outgrowth (CAMPBELL *et al.*, 1965).

Our findings that spore germination is more salt-sensitive in simple than in complex germinating media, seem analogous to the limiting a_w for growth of vegetative bacteria reported by CHRISTIAN (1955) who found that the limiting a_w for growth of *Salmonella oranienburg* was between 0.94 and 0.95 in complex media adjusted with salts and sucrose but between 0.96 and 0.97 in a simpler defined medium (glucose, NH_4^+ and salts) and in which the addition of amino acids and water soluble vitamins extended the growth range to $a_w = 0.95$.

In precise biochemical and biophysical terms the mode of action of high solute concentrations on spores is largely obscure. Certainly solutes may act by binding water molecules and it may be conjectured that availability of water might be essential for dissociation and removal of cytoplasmic structures (spore cement) thought to be involved in maintenance of the dormant spore-state (TANG *et al.*, 1968; GRECZ *et al.*, 1972). On the other hand, ion concentration and water availability may influence enzyme conformation and in this way affect the activity of enzymes involved in germination and outgrowth. Additionally water participates directly in many biochemical reactions and if less water is available such enzyme reactions might be inhibited. Certain hydrolytic enzymes have been reported to play an important role in germination (GOULD *et al.*, 1966; GOULD, 1969; 1970; GOULD & HITCHINS, 1965). At this time all three possibilities mentioned above should be taken into consideration in attempting to explain the molecular mechanism of inhibition of spore germination by increased salt concentrations.

2.2. The effect of low dose irradiation on spore germination

The effect of gamma irradiation on the rate of germination of spores was investigated in 0.1 m*M* or 20 m*M* L-alanine and in a five-fold dilution

of digest broth. A dose of 100 krad caused a reduction in viable count of less than 40% and slightly stimulated the subsequent germination of spores in 0.1 m*M* L-alanine (the reciprocal ratio of germination rates of irradiated to untreated spores was 1.7). In media normally yielding high germination rates (20 m*M* L-alanine or digest broth) no stimulation (or a slight retardation) was observed.

The effect of irradiation and 4.8% NaCl was also studied in 20 m*M* L-alanine, all OD readings being compared with that of the salt-free suspension directly after inoculation and after incubation for 60 min. In the absence of salt germination was practically complete (more than 60% decrease in OD) within 60 min. Samples containing 4.8% NaCl showed insignificant germination, irrespective of whether or not they had been irradiated. Hence the effect of low doses of radiation depends to some extent on the medium used to study germination and/or recovery.

LEVINSON and HYATT (1960) first reported the activation of spores by ionizing radiation and suggested similarities in the action of heat and radiation. GOULD and ORDAL (1968) reported activation of *B. cereus* PX spores after exposure to gamma radiation but did not detect it on spores of *B. cereus* T or *B. subtilis*. In the present investigations the spores were heat activated before irradiation. The additional stimulation of germination by subsequent irradiation observed in the presence of suboptimal concentration of L-alanine was probably the result of damage to spore permeability barriers but in higher concentration of alanine (or broth) the effect seemed to be obviated. That irradiation changes spore permeability may be concluded from GOULD and ORDAL (1968) who reported that irradiated, but not heated, spores germinated more rapidly in *n*-dodecylamine. This is supported by FARKAS and KISS (1965) who reported irradiation induced leakage of intrasporal (cytoplasmic) materials including Ca-DPA.

2.3. The effect of lethal irradiation and/or heat treatment on spore germination and its salt sensitivity

Spore suspensions were irradiated with 1 Mrad in 20 m*M* phosphate buffer or heat treated at 90 °C for 30 min and in addition, lethal combinations of radiation (500 krad) and heat (90 °C, 30 min) were tested in both sequences of application. Germination was studied in 20 m*M* L-alanine or 1 : 5 digest broth each with 0–9.0% NaCl added. The fall in OD of the samples was followed during incubation at 30 °C and the cell state distributions after 2 hours were established microscopically.

Irradiation or heating alone caused falls in the OD of spore suspensions of 11 and 14% resp., under conditions precluding physiological germination (Fig. 2). 500 krad irradiation followed by 90 °C 30 min heat-treatment caused a fall of *ca.* 30%, while the sequence heat then irradiation reduced the OD by

ca. 15%. A further apparent decrease in OD was caused by increasing concentrations of sodium chloride, the essentially parallel characteristics of this fall in OD plotted in Fig. 2 indicating that it was not affected by the pretreatment of the spores.

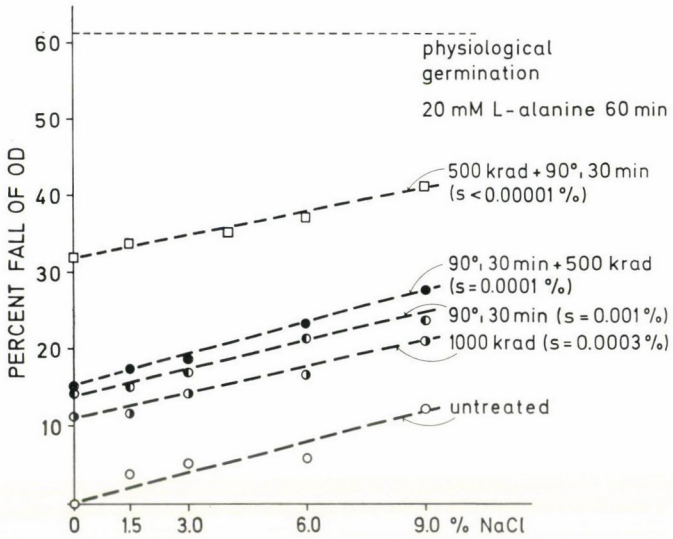

Fig. 2. Decrease of OD of suspensions of *B. cereus* T spores as a result of sporocide treatments and increasing NaCl concentration in the suspending medium. (s = Survival of spores after treatment as established by surface plating on yeast-glucose agar)

Spores which had been irradiated sufficiently to render them non-viable (*i.e.* incapable of forming colonies on YGA) germinated only slightly more slowly than unirradiated spores and this germination was progressively retarded by increasing concentrations of salt (Fig. 3).

Irradiation of spores increased the extent of germination in samples containing higher salt concentrations after two hours of incubation at 30 °C (Fig. 4) although the lethal effect of irradiation was evidenced by reduced numbers of swollen germinated spores in irradiated samples.

The germination of untreated spores, or spores which had been irradiated with 1 Mrad, followed an approximately sigmoidal pattern differing from the response of heated spores and spores treated by combinations of heat and radiation (Fig. 5). After lethal heating or lethal heat plus radiation OD gradually fell with incubation time, but not in a sigmoidal manner. Irradiation followed by heat caused an extensive loss of refractivity of spores, but no further loss of OD occurred.

Increasing concentrations of NaCl decreased the rate of germination in 1 : 5 digest broth of untreated spores or radiation-killed spores (Fig. 6).

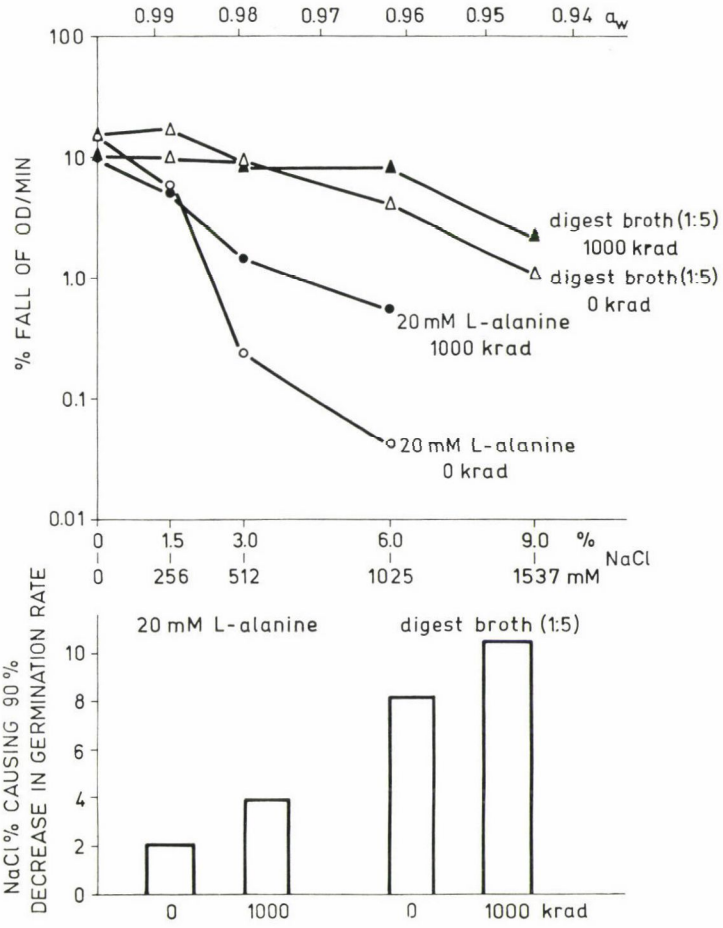

Fig. 3. Top: The effect of lethal irradiation and sodium chloride on the rate of germination of *B. cereus* T spores. Bottom: NaCl % required to decrease germination rate by 90%, as affected by lethal radiation treatment and composition of germination media

The germination rate of spores which had received 1 Mrad was depressed by some 25%, while heating alone or in combination with subsequent radiation reduced the germination rate by *ca.* 93%. While the germination rate of irradiated spores was progressively inhibited by increasing salt concentrations, the germination rates of spores inactivated by heat alone or in combinations with radiation appeared to be independent of the salt concentration in the germination medium. While 1 Mrad rendered spores non-viable in terms of postgerminative development and colony formation, the spore enzyme systems taking part in the germination processes were not inactivated. Lethal heat treatment, and the combinations of heat and irradiation, apparently inactivated the

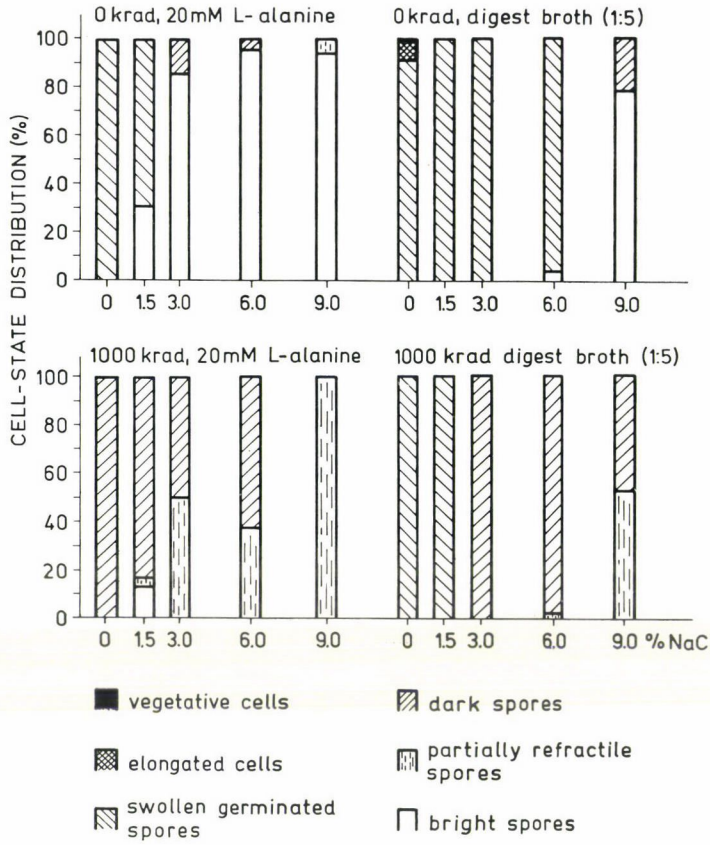

Fig. 4. The effect of NaCl and irradiation on proportions of refractile and germinated *B. cereus* T spores after incubation at 30 °C for 2 hours

germination enzymes. HASHIMOTO and co-workers (1972) reported that heating at 90 °C for 30 min caused a 77% drop in cortex lytic enzyme activity of *B. cereus* T spores, and it seems likely that a similar loss of activity would have occurred here.

That the enzyme systems of microorganisms are less sensitive to radiation than their reproductive capacity, was observed in the early sixties. Dehydrogenase (VAS & FARKAS, 1960, 1961; FARKAS & KISS, 1965) and respiration (LEVINSON & HYATT, 1960) are not inactivated by radiation doses causing several powers of ten reduction in viable count. Other biologically active proteins are resistant to irradiation *e.g. Cl. botulinum* toxin was not substantially inactivated by sterilizing radiation doses either in ground beef irradiated to 4.5 Mrad or within the spores of *Cl. botulinum* irradiated to 3.6–5.0 Mrad (KEMPE & GRAIKOSKI, 1962; GRECZ & LIN, 1966; FERNANDEZ

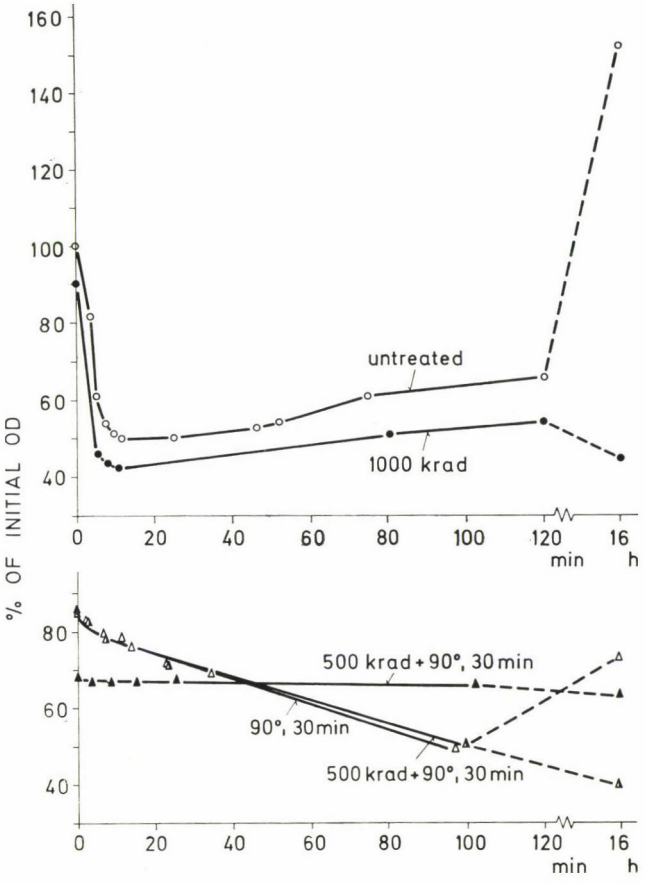

Fig. 5. The effect of irradiation on germination of *B. cereus* T spores in 1 : 5 digest broth. (Initial OD of suspension of untreated spores = 100%)

et al., 1969). Moreover continued synthesis of toxin appeared to occur after 4.5 Mrad radiation within inoculated beef packs when these were incubated at 30 °C for 3–6 weeks even though no viable cells were present (FERNANDEZ *et al.*, 1969).

Irradiation followed by heat treatment caused more drastic changes in the spores than the reversed order of treatment – a difference reported previously in different circumstances by GRECZ (1965).

2.4. Postgerminative development

The salt-sensitivity of postgerminative development was determined by taking the rate of increase of OD during incubation for 16 h of digest broth samples containing 0–3% NaCl. Spore germination was less sensitive to NaCl

than outgrowth and vegetative development (Fig. 7); confirming previous observations (GOULD, 1964; MUNDT *et al.*, 1954; BAIRD-PARKER & FREAME, 1967).

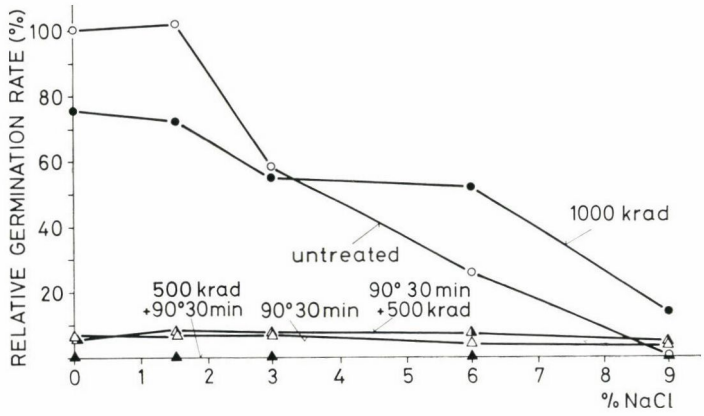

Fig. 6. The effect of NaCl on germination rate in 1 : 5 digest broth of untreated spores of *B. cereus* T and spores previously heated, irradiated, or subjected to combinations of heat plus radiation. (Germination rate of untreated spores in the absence of salt = 100%)

2.5. Colony forming capacity of Bacillus cereus *T spores as a function of irradiation, heat treatment and sodium chloride content of the plating medium*

Spore germination is less sensitive to salt than outgrowth and vegetative cell development (Fig. 3) and this has been recognized by several authors. Gamma irradiation or heating treatment rendered spores increasingly sensitive to post-irradiation inhibition by sodium chloride in the plating medium (ROBERTS *et al.*, 1965; 1966; ROBERTS & INGRAM, 1966). Similarly heat injury of vegetative bacteria results in increased sensitivity to sodium chloride (BUSTA & JEZESKI, 1961; STILES & WITTER, 1965). Thus the influence of heat and irradiation on the salt sensitivity of spores was studied since although synergistic effects are well-known (MORGAN & REED, 1954; KEMPE, 1955) the effect has not been quantitated.

Spores of *B. cereus* T were subjected to a combination of 400 krad gamma irradiation and 90 °C for 12 min heat treatment, in both orders of application, then recovered on YGA with and without NaCl. The colony counts of untreated spores were not affected by up to 2% NaCl in the recovery medium, but spores surviving the combination treatment showed increased salt sensitivity (Fig. 8). The radiation dose used (400 krad) reduced the viability more than the heat treatment (90 °C, 12 min), but the latter produced a bigger increase in salt sensitivity. The combination of heat and irradiation on salt sensitivity proved to be additive or slightly more than additive using recovery

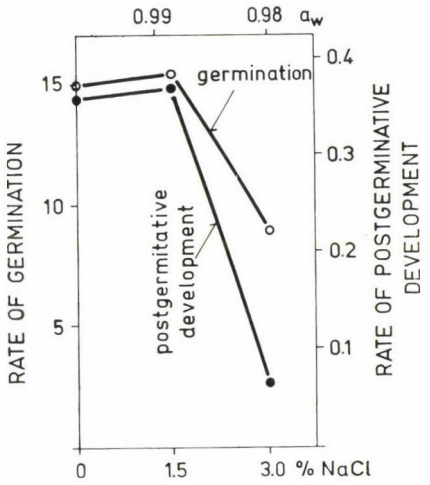

Fig. 7. The effect of NaCl in 1 : 5 digest broth on the rate of spore-germination and post-germinative development of *B. cereus* T. (Rate of germination = per cent fall of OD/min in the steepest part of germination curve. Rate of postgerminative development = the increase of OD during 16 hours of incubation after commencement of germination)

media containing salt. The reverse order of treatment was highly synergistic, resulting in a 500–10 000-fold greater overall reduction in recovery than could be expected from an additive effect. After this treatment, no colonies were

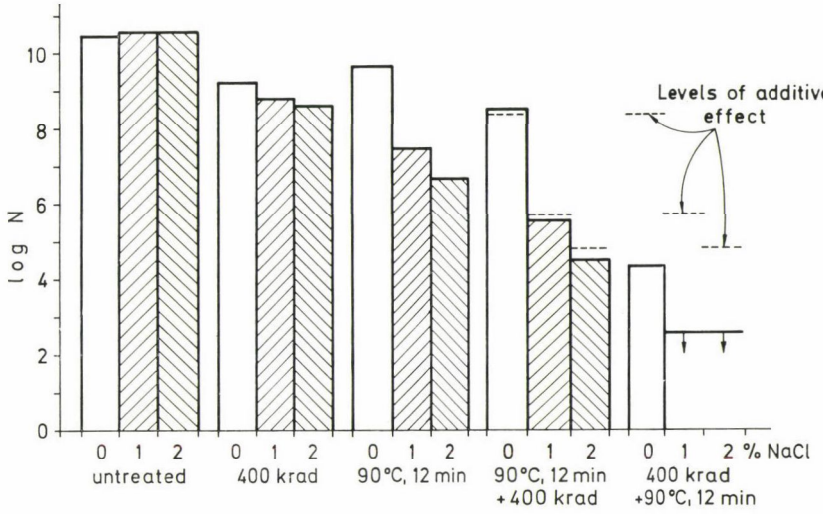

Fig. 8. Effect of heat and gamma-radiation on salt sensitivity of spore recovery on YGA. (The treatments are given in their order of administration, *e. g.* 90 °C, 12 min + 400 krad meant that spores were heated at 90 °C for 12 min first and subsequently irradiated to 400 krad. Recovery of injured spores was evaluated after incubation of plates at 30 °C for 96 hours. The calculated recovery assuming that a combination of radiation plus heat would be additive is indicated by the dotted lines. A value lower than the theoretical indicates synergistic action)

formed on media containing added salt even at the lowest dilution level of the spore suspension.

From these results one might assume that appropriate combinations of heat and water activity could effectively reduce the radiation dose necessary for preservation of foods contaminated with bacterial spores, particularly since the increased salt sensitivity of clostridial spores subjected to combination treatment has already been established (ROBERTS, 1970; FARKAS, 1970a, b); but confirmatory tests on food packs would be essential before extrapolating these data in media. In addition, the nutritional changes and eating quality of such product would also need critical evaluation. Combination processes have already been suggested for spices (FARKAS *et al.*, 1971, 1973a) and Vienna sausages (INCZE *et al.*, 1973; FARKAS *et al.*, 1973b).

Experience has shown that severely damaged spores are more difficult to recover in bacteriological media. A fuller understanding of the nature of the damage produced by combinations of heat and gamma radiation might enable media to be modified to optimise recovery.

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CONTENTS

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CHANGES IN THE QUALITY CHARACTERISTICS OF TOMATO PURÉE DURING SPRAY DRYING

Á. ALPÁRI

(Received April 19, 1975)

Some quality characteristics, such as fat and water-soluble colour values, pH, solids content, acid and sugar content and consistency of tomato purée and of the powder manufactured by spray drying of the purée, were investigated.

Results have shown the fat-soluble colour value to be reduced during spray drying by about 10% on the average, probably as a consequence of the breakdown of lycopene. At the same time an increase of about 25% was observed in the water-soluble colour value of the samples with an average moisture content of about 3.7%, due to caramelization. In powdered tomato juice of a moisture content below the average (*e.g.* below 3%) the increase in the water-soluble colour value may reach 50%. The sugar content of the samples was reduced by about 5%, also due to caramelization. The *Bostwick* value, characteristic of consistency, decreased by about 10% on the average, that is the reconstituted purée was slightly more viscous. The differences between purée and powder in the above characteristics were significant.

Changes in pH and in the acid content were insignificant during spray drying.

Some chemical characteristics of the tomato purée prior to spray drying and of the powdered tomato obtained from it, were studied in parallel. In grading both tomato purée and powder the colour of the product, the pH, solids content, acid and sugar contents as well as the consistency play an important part.

The pigment content is partly fat-soluble, partly water-soluble. The fat-soluble pigment lending its characteristic colour to tomatoes and the products made of them consists of two carotenoids, lycopene and beta-carotene.

According to SZABÓ (1972) about 92–93% of the total fat soluble pigment content is lycopene, while beta-carotene amounts to 7–8% only. The maximum light absorption of lycopene, due to 13 conjugated double bonds, occurs in the visible range at 472 nm. Maximum light absorption with beta-carotene is manifested at 451 nm, since this compound contains only 11 double bonds (LÁSZTITY, 1975).

The water soluble colour value is characteristic of browning in tomato products. Browning may be caused by the *Maillard* reaction, or polyphenoloxidase enzyme activity, but mainly, as a result of heat treatment, caramelization. This may occur during evaporation or sterilization of the purée. During spray drying browning is enhanced by contact with hot air or with the wall of the drying chamber.

SZABÓ (1972) found that during spray drying the value of fat-soluble pigment was reduced by about 10%, while the water-soluble pigment increased by about 80%. The tomato powder investigated by this author contained about 2.3% moisture on the average. She examined 64 tomato purée samples and 62 powder samples.

The aim of this study was to establish the changes in other characteristics during spray drying, on the one hand, and the effect of these characteristics upon the most important parameter of tomatoes, upon their colour, on the other.

The mathematical statistical evaluation of the results permitted of drawing conclusions as to the appropriate frequency of parameter testing.

1. Materials and methods

The NIRO spray drying equipment located at Plant II of the KECSKEMÉT CANNING FACTORY is used to manufacture, from a tomato purée of 28–33% solids content, powdered tomatoes of 3–4% moisture content. During the tomato processing season the tomato purée is fed from the evaporator directly into the NIRO equipment. Out of season, purée preserved in jars or cans is processed.

Atomization of the tomato purée is done by an atomizer disc of about 10 000 rpm. The temperature of the purée reaching the disc is 80–85 °C and it is sterilized.

In the drying chamber the material advances in the same direction as the drying air stream, because tomato purée is a heat sensitive material. The air required for drying is pressed through a filter in the air heating chamber. The filtered air of 140 $\pm$ 1 °C reaches the top of the drying chamber through an air distributor where it contacts the atomized purée. The drying chamber is 12 m high and of 8 m diameter and conical-shaped. The outlet is at the bottom of the chamber. The temperature of the outgoing powder is between 35 and 60 °C, dependent on particle size.

The passage of the material through the evaporator takes about an hour, however, this is only an approximate value because of constant mixing and of the sticking of the powder to the wall of the chamber.

The humid, used air is removed from the chamber and let out in the open by a ventilator.

Electric hammers are fitted to the outside of the drying chamber, in order to help the powder clinging to the inner surface to slide down to the outlet port at the bottom of the chamber. The chamber is jacketed and the air sucked through cools the wall of the cyclone. The major part of the powder goes directly from the drying chamber to a conveyor belt. A substantial part

of the powder is removed with the air stream and is separated from it by the main cyclone.

Filling and packaging in tins or cartons is being carried out in a climatized room, otherwise the highly hygroscopic powder would adsorb moisture and would stick together.

Under normal conditions the equipment produces about 180 kg tomato powder per hour of 3–4% moisture content.

The tomato purée used for spray drying is inhomogeneous to a certain degree. This is because the tomatoes used for processing are cultivated on a large area. In order to account for this heterogeneity, tomato purée batches manufactured from tomatoes grown within the same month on an area of nearly identical climatic conditions (the neighbourhood of Kecskemét and Szeged) were selected for the experiment. Since about 80% of the tomato powder is made of tomato purée preserved in jars or cans, only such product was used. The spray drying of the selected purée took about 3 months.

Several methods are available for the determination of the fat-soluble pigment. Comparative measurements carried out in co-operation with the Analytical Department of the Research Institute of the Canning and Paprika Processing Industries and the Vegetable Production Research Institute have shown the modified *Knorr* method (Food Product Co. Ltd., London) to be the most suitable for colour extraction. According to this method (British Norm) an amount of tomato purée corresponding to 0.1 g solids content, or the same amount of tomato powder, was thoroughly rubbed with 3 ml N-N-dimethyl formamide in a mortar and the fat-soluble pigment was extracted by shaking for 10 min in a shaking apparatus with 50 ml petroleum ether. Then 47 ml of a 2% aqueous sodium chloride solution were added, this was shaken for 5 min and the two phases were separated in a separating funnel. The petroleum ether phase was filtered. The optical density of the filtrate was measured in a 1 cm cuvette at 472 nm against petroleum ether and the value obtained was converted into the value for a solution of 1% solids content ($A^{1\%}_{472nm}$).

To measure the water-soluble pigment content the method of Casoli and Cultrera (1967) proved most satisfactory. An amount of purée corresponding to 1 g of solids, or equivalent amount of powder was weighed, made up to 100 ml with distilled water. It was shaken, allowed to stand for an hour and Kieselgur was added and shaken afresh. The optical density was measured in the filtrate at 380 nm against a blank containing water and Kieselgur. The result was converted for a solution of 1% solids content ($A^{1\%}_{380nm}$).

The colour values, fat- and water-soluble, were measured in a *Bausch & Lomb Spectronic 100* spectrophotometer.

To measure the pH the purée as well as the reconstituted powder were

adjusted to a refractive index corresponding to 5% solids content at 25 °C. Measurements were carried out with an OP-204/1 *Universal* pH meter.

The *Lane–Eynon* method was used to determine the sugar content (GOOSE & BINSTED, 1964). The inverted sugar solution, after clarification, was added, drop by drop, from a burette into the boiling alkaline copper sulphate solution of known concentration in the presence of methylene blue as the indicator.

The acid content was determined by the method laid down in HUNGARIAN STANDARD (1974). Titration was carried out with a 0.1 *n* sodium hydroxide solution in the presence of phenolphtalein indicator.

A *Bostwick* consistometer was used to establish consistency (BONTOVITS *et al.*, 1974). The tomato purée sample was diluted to a refractive index corresponding to 13% solids at 20 °C and was poured into the 5×5 cm sample dish of the *Bostwick* apparatus. After opening the lock the purée was left to spread in the graduated trough for 30 s. Hereafter spreading was read at three points: in the middle and the two sides of the trough. The arithmetical mean of the three values, expressed in cm, is characteristic of the consistency.

To determine the solids content in the purée an *Abbe* refractometer, provided with thermostat, was used at 20 °C; the solids content of the powder was determined by drying in an oven at 70 °C for 6 hours, under vacuum.

Since the determination in the vacuum oven took about 8 hours, the moisture content of the powder was determined also by the rapid *Karl Fischer* method (ERDEY, 1969). This was necessary in order to be able to direct production.

Finally the average ($\bar{x}$), the standard deviation (s) and the standard error ($s_{\bar{x}}$) were calculated for every parameter (KÖRMENDY, 1964). The number of samples required for each permissible error (permissible accuracy) was also calculated and the significance at 95% probability level of related mean values, or deviation was investigated by the *t* or *F* tests.

2. Results

2.1. Colour determination

The colour of the tomato purée and of the powder was determined every 4 hours, the results were converted for a solution of 1% solids content. The fat-soluble colour value ($A^{1\%}_{472nm}$) of tomato purée was found to be between 3.83 and 7.82, the average: $A^{1\%}_{472nm} = 5.830$ (of 325 results).

The fat-soluble colour value belonging to the powder ($A^{1\%}_{472nm}$) varied between 3.33 and 7.04, the average $A^{1\%}_{472nm}$ being 5.240 (of 325 results).

The water-soluble colour value ($A^{1\%}_{380nm}$) of the purée was found to be between 0.18 and 0.49 with an average of $A^{1\%}_{380nm} = 0.340$ (325 results). The

water-soluble colour value of the powder was between 0.27 and 0.79, with an average of $A^{1\%}_{380nm} = 0.420$ (of 325 results).

In the vacuum oven the total solids content was also determined every 4 hours. The total solids content of the powder was 96.634% on the average, varying between 95.44 and 97.88% (325 data).

Solids content determinations by the *Karl Fischer* method were carried out every 3 hours. The average solids content of the powder was found to be 96.362% (436 data).

Since the pH did not change during spray drying it was measured only once a day for the sake of control. The pH of the purée was measured every 4 hours. The pH of the purée was between 4.15 and 4.40, with an average of 4.280 (316 data) while that of the powder was between 4.12 and 4.35, with an average of 4.261 (56 data).

The acid content of the purée and of the powder was established every 16 or every 8 hours. The sugar content was determined daily. The acid content was calculated on dry weight basis and expressed as citric acid. The sugar content was calculated for solids content and expressed as invert sugar.

The acid content of the purée was found to be between 4.59 and 7.86%, with an average of 6.501% (103 data).

The acid content of the powder varied between 5.05 and 7.20%, with an average of 6.591% (207 data).

The total sugar content in the purée was between 51.6 and 65.0%, with an average of 58.11% (58 data).

The total sugar content of the powder varied between 50.1 and 59.0%, with an average of 55.06% (58 data).

The consistency of the infed purée was measured every 4 hours and it was found to be between 8.1 and 16.6 cm, 12.51 cm on the average (329 data).

A series of comparative measurements had been carried out earlier and the consistency of the tomato purée was found to be between 7.8 and 12.0 cm, when diluted to 13% (refr.), 8.8 cm on the average (61 data). The powder obtained from this purée when reconstituted to 13% (refr.), had a consistency of 7.0–11.3 with an average of 7.8 cm (61 data). Thus the reconstituted purée was more viscous than the original.

2.2. Mathematical statistical evaluation of the parameters

2.2.1. Fat-soluble colour value. Changes in the fat-soluble colour value during spray drying are given in Table 1.

Data permit of calculating the required number of tomato purée samples belonging to different standard errors:

325 samples when $s_{\bar{x}} = 0.045$
266 samples when $s_{\bar{x}} = 0.05$
67 samples when $s_{\bar{x}} = 0.1$
17 samples when $s_{\bar{x}} = 0.2$

Table 1

Changes in the fat-soluble colour value ($A^{1\%}_{472nm}$) during spray drying of tomato purée

Product	Number of data (N)	Mean value ($\bar{x}$)	t	t_{crit} P = 95%	Standard deviation (s)	F	F_{crit} P=95%
Tomato purée	325	5.830	11 significant	1.96	0.817	1.26 significant	1
Tomato powder	325	5.240			0.728		

Required number of tomato powder samples belonging to different standard errors:

325 samples when $s_{\bar{x}} = 0.04$
212 samples when $s_{\bar{x}} = 0.05$
53 samples when $s_{\bar{x}} = 0.1$
13 samples when $s_{\bar{x}} = 0.2$

2.2.2. Water-soluble colour value. Changes in the water-soluble colour value during spray drying are shown in Table 2.

Table 2

Changes in the water-soluble colour value ($A^{1\%}_{380nm}$) during spray drying of tomato purée

Product	Number of data (N)	Mean value ($\bar{x}$)	t	t_{crit} P = 95%	Standard deviation (s)	F	F_{crit} P = 95%
Tomato purée	325	0.340	20 significant	1.96	0.051	1.08 significant	1
Tomato powder	325	0.420			0.053		

Required number of tomato purée samples belonging to different standard errors:

325 samples when $s_{\bar{x}} = 0.003$
100 samples when $s_{\bar{x}} = 0.005$
26 samples when $s_{\bar{x}} = 0.01$

Required number of tomato powder samples belonging to different standard errors:

325 samples when $s_{\bar{x}} = 0.003$
112 samples when $s_{\bar{x}} = 0.005$
28 samples when $s_{\bar{x}} = 0.01$

2.2.3. Changes in the pH. Changes in pH during spray drying of tomato purée are shown in Table 3.

Table 3

Changes in the pH during spray drying of tomato purée

Product	Number of data (N)	Mean value ($\bar{x}$)	t	t_{crit} P = 95%	Standard deviation (s)	F	F_{crit} P = 95%
Tomato purée	316	4.280	1.92 non-significant	1.96	0.032	5.2 significant	1.389
Tomato podwer	56	4.261			0.072		

Required number of tomato purée samples belonging to different standard errors:

316 samples when $s_{\bar{x}} = 0.002$
41 samples when $s_{\bar{x}} = 0.005$
11 samples when $s_{\bar{x}} = 0.01$

Required number of tomato powder samples belonging to different standard errors:

200 samples when $s_{\bar{x}} = 0.005$
56 samples when $s_{\bar{x}} = 0.009$
50 samples when $s_{\bar{x}} = 0.01$

2.2.4. Changes in the acid content. Changes in the acid content during spray drying of tomato purée are shown in Table 4.

Table 4

Changes in the acid content during spray drying of tomato purée

Product	Number of data (N)	Mean value ($\bar{x}$)	t	t_{crit} P = 95%	Standard deviation (s)	F	F_{crit} P = 95%
Tomato purée	103	6.501	1.25 non-significant	1.96	0.743	12 significant	1.254
Tomato powder	207	6.591			0.207		

Required number of tomato purée samples belonging to different standard errors:

103 samples when $s_{\bar{x}} = 0.073$
56 samples when $s_{\bar{x}} = 0.1$
25 samples when $s_{\bar{x}} = 0.15$
14 samples when $s_{\bar{x}} = 0.2$

Required number of tomato powder samples belonging to different standard errors:

207 samples when $s_{\bar{x}} = 0.014$
17 samples when $s_{\bar{x}} = 0.05$
5 samples when $s_{\bar{x}} = 0.1$

2.2.5. Changes in the sugar content. Changes in the sugar content during spray drying of tomato purée are given in Table 5.

Table 5

Changes in the sugar content during spray drying of tomato purée

Product	Number of data (N)	Mean value (x)	t	t_{crit} P = 95%	Standard deviation (s)	F	F_{crit} P = 95%
Tomato purée	58	58.11			2.93		
			6	2.98		1.82	1.54
			significant			significant	
Tomato powder	58	55.06			2.17		

Required number of tomato purée samples belonging to different standard errors:

58 samples when $s_{\bar{x}} = 0.38$
35 samples when $s_{\bar{x}} = 0.5$
9 samples when $s_{\bar{x}} = 1.0$

Required number of tomato powder samples belonging to different standard errors:

58 samples when $s_{\bar{x}} = 0.28$
19 samples when $s_{\bar{x}} = 0.5$
5 samples when $s_{\bar{x}} = 1.0$

2.2.6. Changes in the solids content. Changes in the solids content of tomato purée during spray drying are shown in Table 6.

Table 6

Changes in the solids content during spray drying of tomato purée

Product and determination method	Number of data (N)	Mean value (x)	t	t_{crit} P = 95%	Standard deviation (s)	F	F_{crit} P = 95%
Tomato purée	656	31.80	—	—	2.075	—	—
Tomato powder — by gravimetry	325	96.634			6.020		
			0.60	1.96		1.2	1.0
			non-significant			significant	
Tomato powder — by *Karl Fischer*'s method	436	96.362			6.581		

Required number of tomato purée samples belonging to different standard errors:

656 samples when $s_{\bar{x}} = 0.08$
430 samples when $s_{\bar{x}} = 0.1$
108 samples when $s_{\bar{x}} = 0.2$

Required number of tomato powder samples belonging to different standard errors (using the gravimetric method):

3630 samples when $s_{\bar{x}} = 0.1$
908 samples when $s_{\bar{x}} = 0.2$
325 samples when $s_{\bar{x}} = 0.33$

Required number of tomato powder samples belonging to different standard errors (when *Karl Fischer*'s method is used):

4330 samples when $s_{\bar{x}} = 0.1$
1083 samples when $s_{\bar{x}} = 0.2$
436 samples when $s_{\bar{x}} = 0.31$

2.2.7. Changes in consistency. Changes in consistency of tomato purée during spray drying are shown in Table 7.

Table 7

Changes in the consistency during spray drying of tomato purée

Product	Number of data (N)	Mean value (x)	t	t_{crit} P = 95%	Standard deviation (s)	F	F_{crit} P = 95%
Tomato purée	329	12.51	–	–	1.23	–	–
Comparative analysis carried out earlier							
Tomato purée	61	8.8	3.03	2.98	2.00	1.54	1.53
			significant			significant	
Tomato powder	61	7.8			1.61		

Required number of tomato purée samples belonging to different standard errors:

400 samples when $s_{\bar{x}} = 0.1$
100 samples when $s_{\bar{x}} = 0.2$
61 samples when $s_{\bar{x}} = 0.25$

Required number of tomato powder samples belonging to different standard errors:

156 samples when $s_{\bar{x}} = 0.1$
65 samples when $s_{\bar{x}} = 0.2$
61 samples when $s_{\bar{x}} = 0.21$

3. Conclusions

Results of measurements have unambiguously shown that the *fat-soluble* colour value was reduced by 0.5–0.8 units, 0.6 units on the average

(10%) during spray drying. This reduction is probably due to the effect of heat and oxygen upon lycopene and its consequent breakdown.

The *water-soluble* colour value increased by 0.03–0.4 units, 0.08 units on the average (about 25%) in samples of 3.7% average moisture content. This change is due to heat effect and the consequent browning or burning down of the tomato.

The spray drying apparatus was in operation for 5–6 days per week. It was necessary to remove from the wall of the drying chamber the adhering powder once a day (so-called dry cleaning). After 5–6 days a complete stop was necessary and a thorough wet cleaning. During operation 16 electric hammers kept tapping the wall of the chamber to prevent the adhesion of large amounts of powder.

In the course of investigations not to be discussed here in detail, it became evident that the difference in the water-soluble colour value of the purée and the powder increased toward the end of each week. The water-soluble colour value of the powder was between 0.55 and 0.79 by the end of the week, while in the first half it was between 0.27 and 0.55. The reason for this seems to be that dry cleaning did not completely remove the adhering powder particles and they got gradually burnt to the wall. Fresh powder particles adhered to this layer and after some time the adhering layer broke off by its own weight.

From results not to be discussed in detail, it became evident that when the solids content of the powder reached 97% water-soluble colour became about 50% higher than in the purée. This was a consequence of the powder of low moisture content being in contact for a long period with hot air and thus browning was increased.

The difference between the pH of the original purée and the pH of the purée reconstituted from the powder was not significant.

During spray drying a part of the *sugar content* broke down or caramelized, thereby highly increasing the water-soluble colour value.

Tomato purées of low *acid content* (below 6%) and of high sugar content (above 55%) invariably had a high *fat-soluble colour value* ($A^{1\%}_{472nm} = 5.00$ or above) and the same was true for the powder obtained from them. The reason for this phenomenon seems to be that the fruits of which these purées were prepared had been highly insolated and picked fully ripe.

A series of experiments, carried out earlier, has shown the *Bostwick* value, characteristic of consistency, to become reduced by about 10%, that is the consistency improved, as a consequence of spray drying the tomato purée.

The mathematical statistical evaluation of the results has shown a significant difference in the fat- and water-soluble colour value, the sugar content and the consistency of tomato purée and the powder obtained from it. The difference in pH value and acid content was not significant.

Each parameter was measured with adequate frequency, except the solids content of tomato powder. To determine with sufficient accuracy the solids content of the powder by both the gravimetric and the *Karl Fischer* methods a multiple of the normal frequency had to be applied. Thus it seems advisable to build in the spray drying equipment an automatic moisture determination apparatus.

*

The author wishes to express her appreciation of the help rendered in this work by her colleagues in the laboratory of Plant II of the KECSKEMÉT CANNING FACTORY

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INFLUENCE OF THE ZETA POTENTIAL OF COCOA POWDER ON THE STABILITY OF COCOA SUSPENSIONS AND CORRELATION BETWEEN THE ZETA POTENTIAL AND OTHER PARAMETERS

F. HIRSCHBERG and J. TAPODÓ

(Received June 26, 1975)

Twenty cocoa powder samples were analysed by the authors. Apart from the customary analyses sedimentoscopy and zeta potential measurements were carried out. Mathematical statistical evaluation of the results has shown the electrokinetic potential of cocoa particles to significantly affect their capacity to remain suspended. At the same time zeta potential is influenced by some other parameters (pH, ash, *etc.*).

By measuring the zeta potential the authors hope (a) to possess a means for clarifying problems related to cocoa processing (*i.e.* processes occurring during treatment, dissociation phenomena, changes in colour intensity, interrelations during treatment, *etc.*) and (b) to optimize the parameters of cocoa treatment in practice.

The technology of cocoa powder production for cocoa drinks aims at a product of adequate suspension stability.

The properties of cocoa powder are determined by a number of biological, chemical, physical and physico-chemical factors. The biological factors are discussed in detail in a monography by ROHAN (1963) and published by FAO. The book describes the effect of variety, maturity, date of harvesting, *etc.* upon the composition of the cocoa bean. The primary processing operations (fermentation, drying), are also discussed. It is shown, for instance, that the fact whether the bean is located inside the pile, or on its surface during fermentation, is not indifferent. Thus, the cocoa used in the confectionary industry is a heterogeneous material, moreover each factory uses a special mixture of varieties. The technologies applied, the order of operations and the way of their execution all tend to increase heterogeneity. Most of the books on processing technology, *i.e.* those of VIJNOOGST (1963), FINCKE (1965) and MINIFIE (1970) point out that particle size and treatment are important operations in cocoa powder manufacture inasmuch as they affect the stability of the suspension.

The importance of particle size is explained by *Stokes'* law. In this question the literature is unanimous as laid down by KLEINERT (1970).

However, opinions differ as regards the role of the surface of cocoa particles. There is no complete accord concerning the necessity of alkaline treatment. This process was introduced by VAN HOUTEN in 1828. WOLF and co-workers (1963) consider water digestion as efficient as alkaline treatment. Alkaline treatment is preferred by VIJNOOGST (1963). MINIFIE (1970) points

out that the processes occurring upon alkaline treatment are not sufficiently known as yet, but it is certain that not only the neutralization of acids formed during fermentation is involved.

In the opinion of some authors the suspendability of the powder is related to the wetting capacity of the surface. MINIFIE (1970) attributes low wetting capacity to air adsorbed on the surface. To improve the wetting capacity of cocoa powder several inventions were suggested. WHELAN (1970)

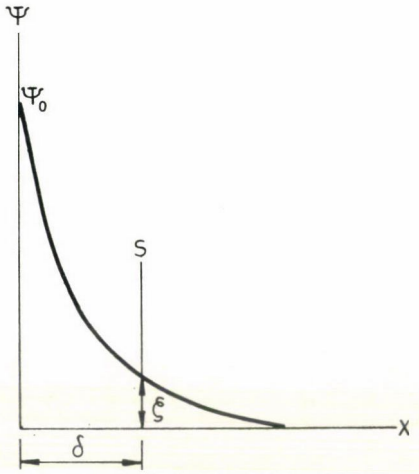

Fig. 1. Electrokinetic or zeta potential. Ψ — potential (not dimensioned); Ψ_0 — surface potential; ζ — zeta potential; δ — thickness of the adhering fluid layer; x — distance to the surface of the material (not dimensioned); S — plane of movement

proposes the treatment of cocoa surface with dioctyl Na succinate at a concentration of 0.1–0.8%, other authors recommend other surface-active agents, such as sugar, lecithin, powdered milk, *etc.* Most of these methods and processes are based on experience. Recent literature adverts to the fact that cocoa powder manufacture is still directed by industrial experience and the research results remain classified.

Little was published so far about the electric surface of cocoa powder. Only the patented invention of MORINAGA CONFECTIONARY Co. Ltd. (1967) utilizes the surface electricity of cocoa powders by taking the powder into spaces of different charges (positive or negative) but mostly in order to achieve agglomeration.

As is well known, zeta potential is developed when a cocoa particle is displaced and the diffuse layer surrounding the particle (sheath of water) bursts open at δ distance from the surface and the particle migrates with its kinetic unit. The electrical potential difference between the cleavage plane (S) and the inside of the liquid phase is the electrokinetic or zeta potential. This is illustrated in Fig. 1.

The role of zeta potential in the extent of hydration or suspendability of the particles is well known, yet in the literature related to the confectionary industry this problem is not discussed. Thus, it seemed obvious to investigate the zeta potential of cocoa powder and approximate by a physico-chemical method the stability of its suspension. We expected to find a correlation between the zeta potential of the cocoa powder and the stability of its suspension, or to find the parameter or parameters correlated with the zeta potential by determining all the parameters usually tested in grading cocoa powders (fat content, ash, alkalinitiy of ash, *etc.*).

In the knowledge of the parameters determining the zeta potential it may become possible to influence them by means of technology thereby increasing the stability of the suspension.

1. Materials and methods

1.1. Materials

All reagents used were of analytical grade and the water distilled, unless indicated otherwise.

Tap water

The hardness of tap water used for the investigations was around 14.8 German degrees (average value for the experimental period).

Cocoa powder

Twenty cocoa powder samples were purchased of which 6 samples were taken on 6 successive days in the first shift at the Budapest Chocolate Factory. The samples were not exposed to alkaline treatment. (In Hungary this treatment is not compulsory.) These samples are numbered 1 to 6 in the tables.

Six samples were taken at the Dessert Confectionary Factory, Budapest, on six consecutive days in the first and second shifts. These samples were exposed to treatment with alkali. The serial numbers of these samples are 7 to 12.

Six cocoa powder samples were taken at the Szerencs Chocolate Factory (Hungary). They were random samples originating from different days of production, taken from the store. The powders were exposed to treatment with alkali during processing. Their serial numbers were 13 to 18.

One cocoa powder was the product of the De Zaan firm, Holland, purchased on the Hungarian market. To this sample belongs serial number 19.

One cocoa powder sample was the product of the Bensdorp firm, Holland, purchased on the Hungarian market. This sample is shown under serial number 20.

Beside the above, 2 samples were purchased from the BUDAPEST CHOCOLATE FACTORY and 2 from the DESSERT CONFECTIONARY FACTORY, Budapest (manufactured at a date different from that of the above samples). These samples were used in the zeta potential measurements with tap water and distilled water (samples marked *a*, *b*, *c*, and *d*).

Bellucci-reagent, the mixture of glacial acetic acid – cc. HCl–water (36:5:9).
α-monobromonaphtalene
glycerol
carbon tetrachloride

1.2. Apparatus

Apart from normal laboratory utensils the following were used:

Sedimentoscope for the study of sedimentation. This is an instrument used in the study of the sedimentation of suspensions. (The instrument was developed at the CENTRAL FOOD RESEARCH INSTITUTE, Budapest.)

The instrument is shown in Fig. 2.

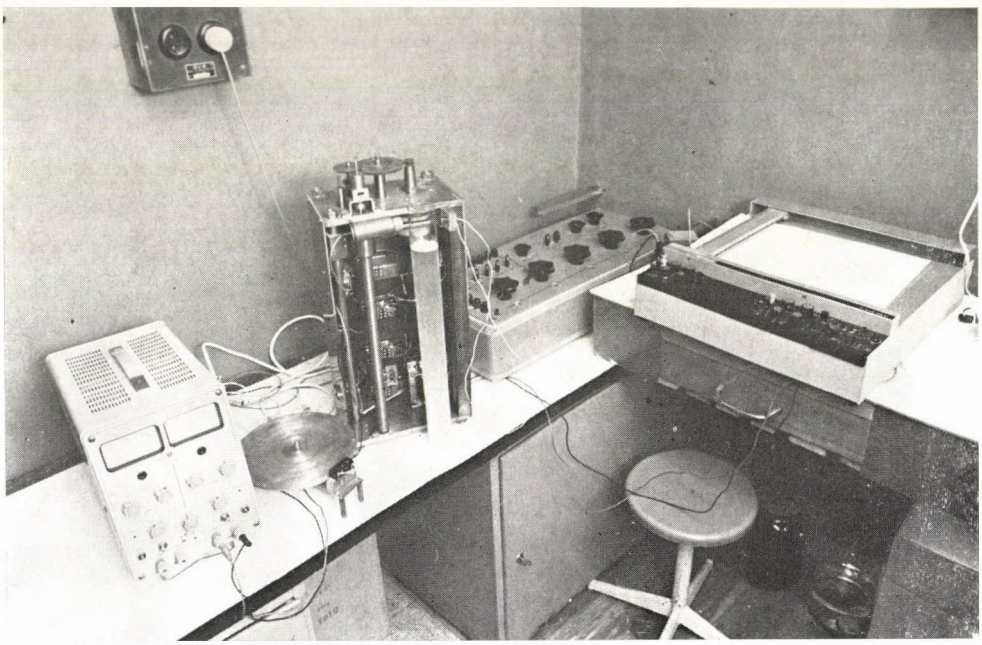

Fig. 2. Sedimentoscope (assembled instrument) (from the left: electricity supply unit rotating timer, sedimentoscope with cuvette, resistance box, *X-Y* decoder)

The assembled instrument consists of the following parts:

— power supply unit

— time switch (disc with microswitch)
— the instrument proper
— resistance box
— X-Y decoder

The principle of the electric circuit of the instrument is shown in Fig. 3.

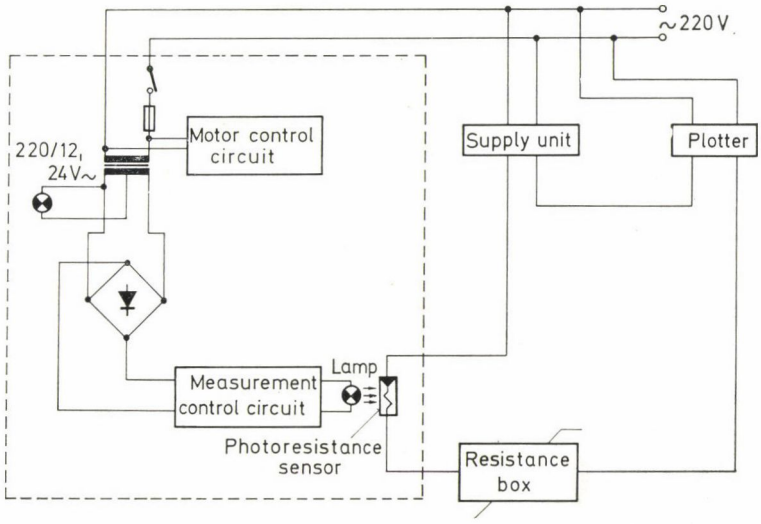

Fig. 3. Principle of the circuitry diagram of the sedimentoscope

The apparatus operates as follows: when the instrument is switched on, the transilluminating lamp lights up. Simultaneously the motor begins to rotate and sets the lamp-spindle into motion. As a consequence the lamp and the photoresistance sensor plate, forming a rigid unit with the lamp, begins to sink. During sinking the cuvette is transilluminated. When the lamp reaches the bottom position, the light goes out and the lamp turning in the opposite direction returns to its initial position. The X-Y decoder records the signals of the photoresistor. By repeating this process at predetermined intervals the record shows the process of sedimentation as a function of space and time.

Riddick Zetameter

This instrument is the product of ZETAMETER Co., New York. The instrument measures the zeta potential of the particles by their electrophoretic speed. A schematic diagram of the instrument is shown in Fig. 4.

By means of the stabilized electric supply unit a definite difference of potential is induced between the two electrodes placed in the suspension. In the electric field, thus formed, the particles start to migrate in the cell. By using diffuse side-light the migration of the particles may be followed on the basis of the *Tyndall*-effect with the microscope. The microscope is fitted with

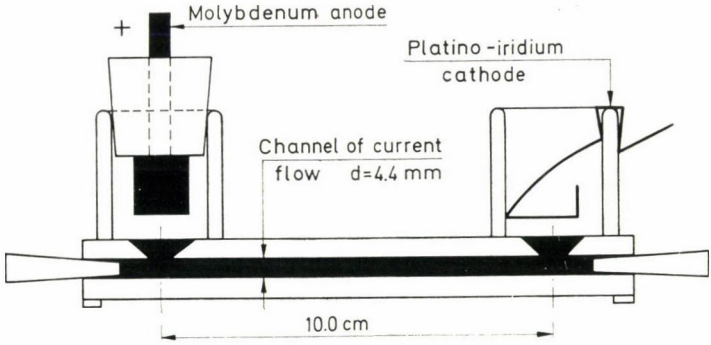

Fig. 4. Schematic diagram of the measuring cell of the *Riddick* zetameter

an ocular micrometer and thus the migration rate of the selected particles may be determined. This value is used to calculate zeta potential by the following equation:

$$\zeta = \frac{4\pi\eta C}{\varepsilon U}$$

where

ξ = zeta potential (CGS Volt)
U = frequency of the field (Volt cm^{-1})
C = migration rate of the particles (cm sec^{-1})
η = viscosity of the suspension (poise)
ε = dielectric constant of the suspension.

Milk separator, 1 000 rpm, made by LABOR, Budapest;
Microscope, ZEISS, with ocular micrometer, or demonstration fitting;
Soxhlet extraction apparatus;
Lovibond tintometer, LOVIBOND Ltd., London;
Incinerator;
Drying oven;
Centrifuge tubes, glass, conical of 10 ml, graduated in 0.1 ml divisions;
Measuring cylinder, 250 ml, of 32–33 mm diameter;
Planimeter, made by MOM, Budapest;
pH-meter, Universal, made by RADELKISZ, Budapest.

1.3. Methods

1.3.1. Sedimentoscope tests. 0.6 g cocoa powder is suspended, possibly free of lumps, in 100 ml water of 77–80 °C. The suspension is washed with

500 ml water of room temperature into a 750 ml flask, cooled to room temperature, the pH of the suspension is adjusted (if required), it is thoroughly shaken and poured into a measuring cuvette. The cuvette is placed in the instrument and the first measurement is carried out immediately. The line thus obtained is the "suspension line" (L_s). Then the instrument is operated at definite retention periods for a definite time.

After the final measurement or prior to the start the line given by the cuvette when filled with water is recorded on the same diagram. The line thus obtained is the "water line" (L_w).

With the advance of sedimentation the diagram (particularly its initial phase) deviates from the suspension line to approach the water line. The extent of approachment depends on the rate of sedimentation. The concentration of the suspension at a given point is characteristic of the distance of the point from the water line, while the area enclosed by the diagram and the water line is characteristic of the whole cuvette and this is the clearance percentage ($C_L\%$).

$$C_L\,\% = \frac{S_b - S_i}{S_b}\,100$$

where

S_b = basic area, the area enclosed by the water line and the suspension line

S_i = the area between the diagram recorded at time "i" and the water line.

The sedimentoscope diagram thus obtained has no physical dimensions. Nevertheless, it may be used for some indirect calculations. The vertical axis shows light absorption, the horizontal axis the length of the cuvette (the switch-on point near the origin stands for the top of the cuvette and the end-point on the right for the bottom of the cuvette).

The measurement of the area may be expediently carried out with a planimeter.

These measurements were performed on the cocoa samples in tap water as well as in distilled water.

Figs. 5 and 6, resp., illustrate a sample of high and low floating capacity.

If the pigment content extracted during measurement is needed to be taken into account, then at the end of measurement the cocoa particles are separated by filtering, or by centrifuging and a diagram is plotted on the same record with the clarified solution.

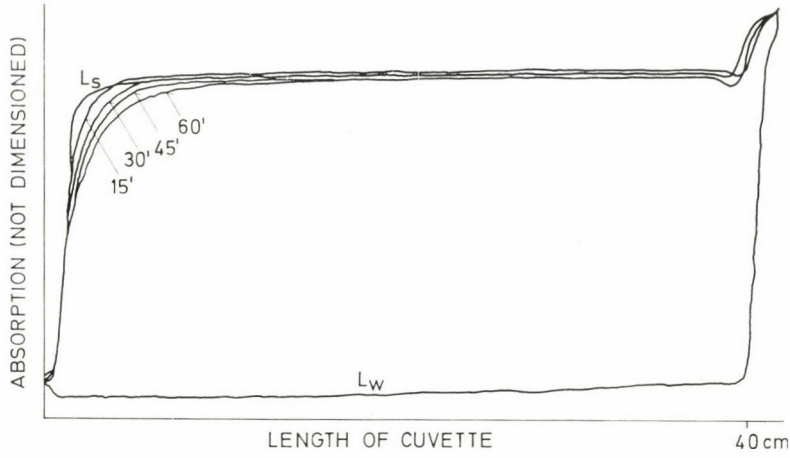

Fig. 5. Sedimentoscope diagram of the suspension of high-quality cocoa powder (0.1% suspension, pH = 5.2). L_s = suspension curve (0 min); L_w = water curve; sedimentograms (15, 30, 45, 60 min) prepared with 15 min retention time

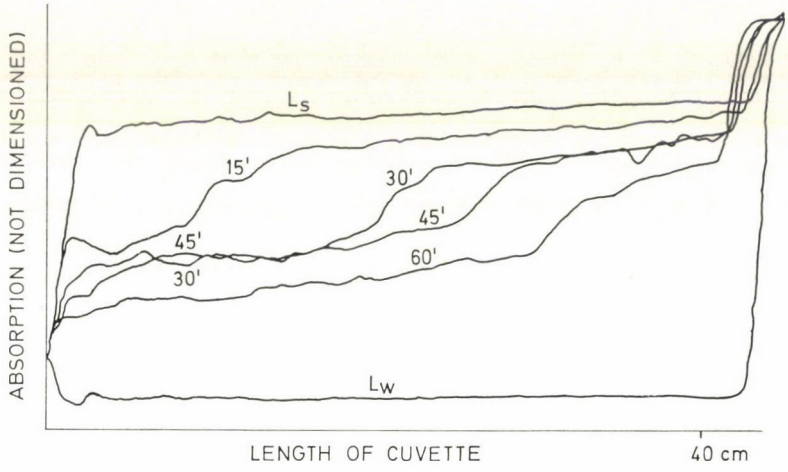

Fig. 6. Sedimentoscope diagram of the suspension of a low-quality cocoa powder (0.1% suspension, pH = 5.2). L_s = suspension curve (0 min); L_w = water curve; sedimentograms (15, 30, 45, 60 min) prepared with 15 min retention time

1.2.3. Sedimentation tests

1.3.2.1. Preparation of the suspension. — Ten g of cocoa powder are thoroughly mixed with 30 ml liquid (distilled water, tap water or milk) then 200 ml of the same liquid is added, taking care to avoid lump formation. The suspension is cooled to room temperature and shaken before use.

1.3.2.2. Sedimentation in centrifuge tube with water, without centrifuging. — Of the suspension prepared according to para. 1.3.2.1. with distilled or tap

water, 10 ml are transferred into a centrifuge tube and the volume of the sediment determined after one hour.

1.3.2.3. Sedimentation in milk in a graduated cylinder. — Of the suspension prepared according to para. 1.3.2.1. with milk, 250 ml are filled into a graduated cylinder and after 10 min the volume of the sediment is read in ml.

1.3.3. Determination of the fat content. The international method OFFICE INTERNATIONAL DU CACAO ET CHOCOLAT, 8a/D/1952) was used.

1.2.4. Determination of ash content. The international method (OFFICE INTERNATIONAL DU CACAO ET CHOCOLAT, 4a/D/1952) was used.

1.3.5. Determination of ash alkalinity. The international method (OFFICE INTERNATIONAL DU CACAO ET CHOCOLAT, 9-D/1961) was used.

1.3.6. Determination of D number (TAPODÓ, *1968*). This is a rapid method for the determination of the degree of dispersity of cocoa powders. An amount of 0.25 g cocoa powder is weighed and 1.5 ml α-monobromo naphtalene are added and mixed for 4 min while moderately heated. One drop of the mixture is placed on a microscopic slide and covered with a covering glass. The maximum diameters of the largest particles in 36 fields of vision, evenly distributed, are measured by means of an ocular micrometer. The average of the values thus measured, in μm, is considered the D number.

1.3.7. Determination of husk content (TAPODÓ, *1973a, b*). Cocoa powder equivalent to 1.17 g fat-free cocoa mass is weighed and refluxed with 25 ml *Bellucci*-reagent. When cold it is shaken with carbon tetrachloride. Two ml of glycerol are added to 2 ml of the aqueous phase. Of this 9.5–10.5 mg are accurately weighed on a slide, covered with a covering glass. The spirals seen in 10 rows at 400-fold magnification are counted. Distance between rows should be 1.6 mm. The husk content is calculated as follows:

$$\text{Husk } \% = 0.05\,S - 0.9$$

where S = the number of spirals counted.

1.3.8. Determination of zeta potential. In accordance with specifications a cocoa suspension of 0.01% is used in distilled or tap water.

1.3.9. Measurement of conductivity. The conductivity of a 0.01% cocoa powder suspension in distilled water is measured with the zetameter as described in para. 1.2.

1.3.10. Lovibond value. The *Lovibond* value (in the tristimulus system) of the cocoa powders was determined with a *Lovibond Tintometer*.

1.3.11. Evaluation of results. The average and standard deviation of zeta potential measurements was determined by the groups of mathematicians at the SCIENTIFIC RESEARCH INSTITUTE FOR WATER SUPPLY MANAGEMENT on the basis of the *Fortran* program by means of the computer of the Institute. The evaluation of the distribution of individual measurements was carried out at the same institute, as follows.

1.3.11.1. Establishment of the distribution pattern (SZIJGYÁRTÓ, 1966; LICSKÓ, 1974). — The density distribution of a population may be symmetric (normal) or asymmetric (called gamma distribution). While the normal distribution may be characterized by two parameters, the average ($\bar{x}$) and the deviation (σ), gamma distribution has three parameters: x_0, λ and k. x_0 is the value of the independent variable at which the value of the dependent variable is equal to zero prior to $-\infty$. It is calculated as follows:

$$x_0 = \bar{x} - 2\frac{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n}}{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^3}{n}}$$

where

x_i = the value of the i-th member
n = number of samples.

The other two parameters may be derived from the *central momentum* generally used with the probability theory. Calculation:

$$\lambda = \frac{\bar{x} - x_0}{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n}}$$

and

$$k = \frac{(\bar{x} - x_0)^2}{\sum_{i=1}^{n}\frac{(x - \bar{x})^2}{n}}$$

To determine the distribution pattern also the so-called third central momentum is necessary. This is sometimes called the asymmetry coefficient of the density function. It is calculated as follows:

$$\mu_{3,n} = \frac{1}{n}\left[\sum_{i=1}^{n}\xi_i - \left(\frac{1}{n}\sum_{i=1}^{n}\xi_i\right)\right]^3$$

where

$\mu_{3,n}$ = the third central momentum
ξ_i = i-th member of the sample.

In the knowledge of the above the following criteria may be set up:

$$x_0 > \xi_1 \text{ and } \mu_{3,n} \leq 0 \tag{1}$$

ξ_1 = is the lowest member of the sample (in this case the lowest zeta potential measured in the sample).

Further, if

$$k > 15 \tag{2}$$

the empirical distribution may be approximated by normal distribution, marked $N(\bar{x}, \sigma)$, or else by gamma distribution, marked $\Gamma(x_0, \lambda, k)$.

1.3.11.2. Investigation of homogeneity. Two samples are called homogeneous if they originate from the same statistical population and this may be proven by mathematical statistical calculations. Or in other words two samples are homogeneous, if the distribution of their characteristic values (in this case their zeta potential) is statistically identical. To decide this, the *Kolmogorov–Smirnov* method, generally used in probability calculations, was applied. Equations are as follows:

$$p = 100\,[1\text{–}L(z)]$$

$$z = d_{n,m}\sqrt{\frac{mn}{m+n}}$$

where

p = value characteristic of homogeneity as %
$L(z)$ = value obtained by the *Kolmogorov* distribution function on the basis of z value
m, n = number of compared samples
$d_{n,m}$ = maximum difference of distribution functions measured in identical zeta potential intervals.

Condition of the criterion of homogeneity: $p > 5\%$

2. Results

Except for the zeta potential measurements, results represent the average values of three measurements. The number of zeta potential measurements are given separately.

Table 1

Zeta potential of cocoa powder samples

Number of sample	Zeta potential, mV		Number of measurements
	average	standard deviation	
1	−23.8	2.7	62
2	−23.6	2.0	65
3	−22.5	1.9	61
4	−25.2	4.0	66
5	−27.1	3.7	66
6	−24.1	2.2	65
7	−26.4	2.6	62
8	−25.4	2.8	65
9	−24.8	2.6	67
10	−23.8	1.9	65
11	−28.7	3.3	68
12	−22.4	2.7	62
13	−23.9	1.9	64
14	−23.5	2.1	67
15	−24.2	2.5	67
16	−22.3	2.4	65
17	−23.0	2.4	65
18	−22.8	2.0	68
19	−29.8	3.4	68
20	−29.2	3.4	65

2.1. Zeta potential of cocoa powder samples

2.1.1. Zeta potential of samples of different origin in distilled water. The average and standard deviation of the zeta potential of samples Nos. 1 to 20 (para. 1.1) are given in Table 1.

As it may be seen in the table cocoa powders possess a negative zeta potential and the values differ. The high negative zeta potentials of Hungarian sample No. 11 and of the two Dutch samples is particularly striking.

Table 2 contains the results of comparison by the F test and Student's t test of all data belonging to one sample to those of all other samples (at probability level 95%).

Differences in standard deviations and t tests are significant.

Table 2

Test of significance of the zeta potential of cocoa powder samples

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	▲	✳	✳	✳	✳	–	–	–	–	–	–	–	✳	✳	–	–	–	✳	✳	✳
2	O	▲	–	✳	✳	–	✳	✳	✳	–	✳	✳	–	–	–	–	–	–	✳	✳
3	⊛	O	▲	✳	✳	–	✳	✳	✳	–	✳	✳	–	–	✳	✳	✳	–	✳	✳
4	⊛	⊛	⊛	▲	–	✳	✳	✳	✳	✳	✳	✳	✳	✳	✳	✳	✳	✳	–	–
5	⊛	⊛	⊛	O	▲	✳	✳	✳	✳	✳	–	✳	✳	✳	✳	✳	✳	✳	–	–
6	⊛	O	O	O	⊛	▲	–	✳	–	–	✳	–	–	–	–	–	–	–	✳	✳
7	⊛	⊛	⊛	⊛	⊛	O	▲	–	–	✳	–	–	✳	–	–	–	–	✳	✳	✳
8	O	⊛	⊛	O	⊛	⊛	O	▲	–	✳	–	–	✳	✳	–	–	–	✳	–	–
9	O	⊛	⊛	O	⊛	O	O	O	▲	✳	✳	–	✳	–	–	–	–	✳	✳	✳
10	O	O	O	⊛	⊛	O	⊛	⊛	⊛	▲	✳	✳	–	–	✳	✳	✳	–	✳	✳
11	⊛	⊛	⊛	⊛	O	⊛	O	⊛	⊛	⊛	▲	–	✳	✳	✳	✳	✳	✳	–	–
12	O	⊛	⊛	⊛	⊛	O	O	⊛	O	⊛	⊛	▲	✳	✳	–	–	–	✳	✳	✳
13	O	O	O	⊛	⊛	O	⊛	⊛	⊛	O	⊛	⊛	▲	–	✳	✳	✳	–	✳	✳
14	O	O	O	⊛	⊛	O	⊛	⊛	O	O	⊛	⊛	O	▲	–	–	–	–	✳	✳
15	O	O	O	⊛	⊛	O	O	O	O	O	⊛	O	O	O	▲	–	–	✳	✳	✳
16	O	O	⊛	⊛	⊛	O	⊛	⊛	O	⊛	⊛	O	⊛	O	O	▲	–	–	✳	✳
17	O	O	O	⊛	⊛	O	⊛	O	O	⊛	⊛	O	⊛	O	O	O	▲	✳	✳	✳
18	⊛	O	O	⊛	⊛	O	⊛	⊛	⊛	O	⊛	O	O	O	⊛	O	O	▲	✳	✳
19	⊛	⊛	⊛	⊛	O	⊛	⊛	⊛	⊛	⊛	O	⊛	⊛	⊛	⊛	⊛	⊛	⊛	▲	–
20	⊛	⊛	⊛	⊛	O	⊛	⊛	⊛	⊛	⊛	O	⊛	⊛	⊛	⊛	⊛	⊛	⊛	O	▲

– difference not significant on the basis of the F test

✳ difference significant on the basis of the F test (probability level 95 %)

O difference not significant on the basis of the t test

⊛ difference significant on the basis of the t test (probability level 95 %)

Since, judged by the distribution study, results of zeta potential measurements most frequently follow the gamma distribution pattern, homogeneity was tested by the *Kolmogorov–Smirnov* distribution function. Results are shown in Table 3.

Table 3

Homogeneity test of the cocoa powder samples

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	●	91.3	18.6	11.8	0.0	25.0	0.0	0.0	0.1	52.8	0.0	9.3	10.7	67.8	22.1	5.9	1.4	57.7	0.0	0.0
2	*	●	2.1	4.7	0.0	43.6	0.0	1.7	3.4	99.3	0.0	8.0	38.0	99.9	42.8	0.2	2.9	19.4	0.0	0.0
3	*	—	●	0.0	0.0	0.1	0.0	0.0	0.0	0.4	0.0	72.8	0.0	0.8	0.0	99.9	80.8	92.3	0.0	0.0
4	*	—	—	●	0.2	11.8	0.1	30.4	56.1	18.6	0.0	0.5	5.9	6.5	83.7	0.0	1.7	0.2	0.0	0.0
5	—	—	—	—	●	0.0	13.6	2.6	0.3	0.0	10.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	*	*	—	*	—	●	0.0	7.2	13.6	15.6	0.0	1.0	35.3	34.0	86.5	0.0	0.1	2.0	0.0	0.0
7	—	—	—	—	*	—	●	2.6	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	—	—	—	*	—	*	—	●	96.4	2.9	0.0	0.0	1.5	2.7	21.2	0.0	0.0	0.0	0.0	0.0
9	—	—	—	*	—	*	—	*	●	13.6	0.0	0.0	15.6	7.6	34.0	0.0	0.0	0.0	0.0	0.0
10	*	*	—	*	—	*	—	—	*	●	0.0	1.9	13.0	99.9	20.8	0.0	2.7	5.6	0.0	0.0
11	—	—	—	—	*	—	—	—	—	—	●	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	34.0
12	*	*	*	—	—	—	—	—	—	—	—	●	0.9	8.8	2.1	42.1	62.8	48.1	0.0	0.0
13	*	*	—	*	—	*	—	—	*	*	—	—	●	28.1	42.1	0.0	0.2	0.5	0.0	0.0
14	*	*	—	*	—	*	—	—	*	*	*	—	*	●	59.4	0.0	1.2	9.3	0.0	0.0
15	*	*	—	*	—	*	—	*	*	*	—	—	*	*	●	0.0	2.1	1.4	0.0	0.0
16	*	—	*	—	—	—	—	—	—	—	*	—	—	—	—	●	24.0	45.1	0.0	0.0
17	—	—	*	—	—	—	—	—	—	—	*	—	—	—	—	*	●	25.0	0.0	0.0
18	*	*	*	—	—	—	—	—	—	*	*	—	—	*	—	*	*	●	0.0	0.0
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	●	66.1
20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*	●

Numbers stand for p % values

* > p = 5 %

— < p = 5 %

As it can be seen, homogeneity exists between the zeta potential distribution of some of the samples, while between other samples it is not mani-

fest. It is particularly interesting that in several cases homogeneity was not manifest in samples of the same origin, while between samples of different origin it existed.

2.1.2. Zeta potential of cocoa samples in distilled and tap water, resp. The zeta potential of four cocoa powders was determined in distilled and in tap water. Results are shown in Table 4.

The results in the table prove that the absolute value of the zeta potential of all cocoa powder samples was higher in distilled water than in tap water. The differences within individual samples were significant at probability level 0.05%.

Table 4

Comparison of the zeta potential of cocoa powder in distilled and in tap water

Sample	Zeta potential, mV						Significance*
	in distilled water			in tap water			
	average	standard deviation	number of mesure-ments	average	standard deviation	number of mesure-ments	
a	−23,2	2.0	68	−9.7	1.3	72	+
b	−24.1	2.2	73	−7.3	1.2	68	+
c	−22.0	2.5	67	−9.0	1.1	68	+
d	−25.0	3.0	67	−10.3	1.5	68	+

* at 95% probability level

2.2. Comparison of the zeta potential of the cocoa samples with other parameters

In order to compare the results to the zeta potencial values, other parameters of the samples were also determined. The results belonging to samples Nos. 1 to 20 are summarized in Table 5.

The zeta potential values seen in this table are identical with those in Table 1. The lowest row shows the correlation coefficient of the given parameter and the absolute value of zeta potential. The negative sign of the zeta potential value was omitted in order to simplify interpretation of results.

The results in the table show that the correlation between zeta potential and some of the parameters is close, with others medium, with some others weak, while there are parameters which show practically no correlation (*i.e.* husks). It may also be seen that with certain parameters the correlation coefficient is negative, with others positive. In other words the correlation between the zeta potential and some of the parameters is direct, in others inverse.

Table 5

Parameters of the cocoa

Serial number of sample	Moisture content %	Fat content %	Ash %	Alkalinity of ash	pH	"D" number	Husk content %	*Lovibond* values		
								blue	yellow	red
1	2.19	22.11	5.11	17.30	5.70	111.3	2.65	3.7	7.5	8.0
2	2.06	22.65	5.11	18.34	5.56	109.4	2.65	4.3	9.0	8.5
3	2.85	21.29	5.22	18.57	5.48	112.8	3.95	3.3	9.0	7.4
4	2.01	22.29	5.26	18.48	5.62	109.1	3.55	3.3	7.7	7.9
5	2.37	22.22	5.29	18.35	5.82	97.7	3.70	3.5	8.0	7.3
6	2.16	22.80	5.22	17.47	5.54	119.1	4.05	4.0	8.4	7.8
7	3.76	21.08	5.65	20.58	5.91	105.0	2.65	3.5	8.5	8.5
8	3.61	20.06	5.06	19.02	6.60	114.8	2.40	2.3	6.0	6.8
9	4.29	21.30	5.28	19.62	5.76	102.2	2.15	3.4	8.0	7.8
10	3.91	21.30	5.72	17.53	6.53	99.0	2.20	3.5	8.4	8.4
11	3.84	20.62	6.24	17.88	5.75	110.5	2.80	3.5	7.3	7.6
12	3.91	20.99	6.02	18.16	6.36	110.0	2.80	3.6	7.6	8.0
13	3.18	25.53	4.48	18.36	4.64	105.0	0.65	2.7	6.2	6.5
14	3.11	24.96	4.39	18.60	4.63	106.2	0.86	2.5	5.5	6.2
15	3.07	24.48	4.50	18.16	4.65	102.5	1.94	3.7	7.8	8.6
16	3.33	25.06	4.27	18.70	4.67	121.4	1.00	3.1	6.5	7.0
17	3.39	24.70	4.34	19.85	4.77	105.2	1.55	3.2	6.4	7.5
18	3.18	22.14	4.33	19.38	4.56	109.4	0.67	3.0	6.7	7.4
19	3.32	22.88	7.70	20.26	7.26	71.0	1.00	4.7	9.0	9.2
20	3.21	22.94	7.17	20.24	6.81	73.4	1.50	3.2	7.4	7.5
Correlation coefficient with zeta potential		−0.240	0.805	0.277	−0.626	0.721	0.015	0.281	0.295	0.368

Data show the mean values of three measurements, except for the zeta potential

powder samples

Conductivity μS	Sediment in milk		Sedimentoscope tests (within 60 min)				Zeta potential mV
	graduated cylinder ml	centrifuge tube ml	in distilled water	in tap water	in distilled water	in tap water	
			area below the curve, cm²		% clearance		
12.4	2.2	0.30	21.46	23.85	28.12	28.31	−23.8
11.3	1.0	0.28	22.07	24.02	37.48	44.55	−23.6
11.3	1.7	0.33	21.83	24.66	33.90	43.28	−22.5
13.0	2.0	0.27	23.42	23.56	36.61	49.81	−25.2
13.3	1.8	0.25	23.64	23.04	34.85	47.95	−27.1
10.4	3.2	0.29	20.26	21.50	46.39	38.78	−24.1
14.0	2.0	0.27	24.60	25.77	10.57	13.00	−26.4
11.2	2.0	0.21	24.92	26.54	4.60	19.35	−25.4
11.2	1.0	0.20	24.31	26.54	11.17	15.35	−24.8
10.2	2.8	0.17	23.16	25.35	5.46	23.42	−23.8
12.4	2.7	0.18	23.91	26.60	7.38	12.55	−28.7
14.4	2.8	0.20	22.95	26.73	15.84	23.47	−22.4
10.3	1.0	–	23.91	24.44	22.85	29.31	−23.9
10.2	1.3	0.13	21.52	23.73	34.94	33.41	−23.5
10.2	1.0	0.10	21.22	25.41	39.80	27.80	−24.2
10.3	1.3	0.13	22.77	25.82	17.49	31.80	−22.3
11.2	1.0	0.20	22.81	23.04	36.35	44.01	−23.0
10.3	1.7	0.20	22.59	23.99	27.85	38.20	−22.8
15.5	0.0	0.00	23.27	28.76	2.27	3.67	−29.8
14.4	0.7	0.05	25.17	28.63	1.84	2.70	−29.2
0.669	−0.252	−0.509	−0.508	0.545	−0.527	−0.629	

The number of zeta potential measurements are given in Table 1

3. Conclusions

As can be seen in Table 1, all the cocoa powder samples, whether predigested (Nos. 7 to 18) or not (Nos. 1 to 6), have a negative zeta potential, so have the Dutch samples as well. Thus the presumption, that the conventional digestion technology causes a specific ion adsorption and thereby changes the sign of zeta potential has to be dropped. The investigations have shown the alkaline treatment to promote dissociation of surface particles.

A significant difference was found in the zeta potentials of the cocoa powder samples investigated. Particularly exceptional zeta potentials were mesured in the Dutch samples and the Hungarian No. 11 sample. Table 2 permits of the same conclusion showing at the same time that significant differences may exist between samples originating from the same factory (*e.g.* Nos. 9 and 11) as regards the F test and *Student*'s t test of the zeta potentials.

The mathematical statistical analysis of the data has shown that the zeta potential values of cocoa powder particles within one sample are not of normal distribution, but rather follow the three-parameter gamma distribution pattern. Thus it seemed advisable to determine the homogeneity of the samples by the *Kolmogorov–Smirnov* distribution function. The results of these investigations are given in Table 3 and show that differences in distribution occur between samples originating from the same factory. On comparing the data in Table 2 and 3, samples are found with non-significant differences in the average and deviation of their zeta potentials, while the distribution of the zeta potential of their particles does not follow the same pattern. Thus, whichever of the evaluation methods is taken into consideration it may be concluded that the zeta potential of cocoa powders is influenced by the quality of technological operations. This may account for the fact that the cocoa powders manufactured during two subsequent shifts, presumably by the same technology and from the same raw material, were of different quality.

It is of interest to compare the zeta potential of cocoa powders as obtained in distilled water and in tap water. As it may be seen in Table 4 the zeta potential of cocoa powders suspended in tap water is substantially lower than those suspended in distilled water (Table 5). This observation is in conformity with the results of the sedimentation experiments where suspensions in distilled water were found more stable. From this it may be concluded that divalent ions present in tap water aggregate the cocoa particles. Considering that milk contains a fair amount of polyvalent ions, this kind of effect has to be taken into account in cocoa drinks, too.

From the data in Table 5 only data showing correlation between zeta potential and other parameters are discussed in the present paper. For the sake of simplification only the absolute value of zeta potentials is used in the calculations.

The high correlation coefficients, between ash and zeta potential on one hand and pH and zeta potential on the other, as seen in Table 5, prove the importance of alkaline treatment.

The importance of fineness of comminution is proven by the high correlation value observed between zeta potential and "D" number. At the same time a belief of old standing is eliminated by this study, namely that the husk content plays an important role in the suspendability of cocoa powders.

The correlation between the alkalinity of ash, the fat content and the *Lovibond* value of cocoa powders and their zeta potential was not so close. The correlation with these parameters was so loose that conclusions could not be drawn.

In our opinion the most important of our observations is the correlation between zeta potential and the sedimentation capacity of cocoa powders. Relevant observations could not be found in the literature.

It has been shown that a negative correlation exists between the absolute value of zeta potential and sedimentation, whatever method is used to study the latter. This means that a high zeta potential reduces the sedimentation capacity. In other words the technology of cocoa powder production has to be modified to produce cocoa powder of high (negative) zeta potential thereby ensuring the stability of the suspension.

Though many problems remained open or were raised in the course of this study (optimum value of zeta potential, surface electricity of particles not measurable with the *Riddick* apparatus, *etc.*), yet it is hoped by the authors, that they succeeded in elucidating the background of the floating capacity of cocoa powder.

By gaining a deeper insight into the relations between zeta potential and other parameters, through the analysis of the electric properties of the particles becomes possible to optimize the production technology of cocoa powder. To achieve this it may become important to measure the electrokinetic potential of cocoa powders by other methods (sedimentation potential, electroosmosis, *etc.*) or even develop an instrument for this purpose.

*

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A STUDY INTO THE KEEPING QUALITY OF CARROT VARIETIES

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The keeping quality of carrot varieties *(Amsterdamer Treib, Gonsenheimer Nantoise, Flakker, Fertődi vörös* and *Chantenay)* was studied (at 9–11 °C and 70–85% RH) with reference to spoilage, weight loss, consistency, solids content and sensory value.

As regards spoilage, weight loss and change of consistency, variety *Chantenay* proved to be of the highest keeping quality and *Fertődi vörös* of the lowest.

Varieties *Amsterdamer Treib, Gonsenheimer* and *Flakker* were also of a good shelf-life.

In the solids content of the samples no or very little change was observed during the storage period of 11 weeks.

The sensory quality of the samples was studied in the raw and cooked state on 0 day and after 5 and 11 weeks storage. Of the sensory characteristics colour, flavour and smell were investigated.

After an 11-week storage period there was no difference between the samples. Only variety *Amsterdamer Treib* formed an exception, having a better colour than the other samples.

As with every horticultural produce, for successful storage of carrots the following background information is essential:

1. properties characteristic of the variety
2. the circumstances of cultivation
3. method of harvesting (manual or mechanical).

Properties of the variety and resistance to diseases influence not only the growing but also the keeping qualities.

Opinions differ as to the effect of conditions of growing upon storage behaviour. HÁJAS (1972) found that the keeping quality was affected by watering the plant during growth, by the rain-fall in the year of cultivation, by the structure and supply of nutrients in the soil. Fertilizers do not seem to affect keeping quality (ZIEGLER & BÖTTCHER, 1966). However, nitrogen fertilization forms an exception, because it unfavourably affects the activity of tissue enzymes and the moisture retaining capacity of the cells.

DERBYSHIRE (1971) studied carrot variety *Chantenay* during several years and came to the following conclusions as regards their keeping quality:

— keeping quality is influenced by the method of harvesting, by the year of cultivation, by washing of the roots, by the soil and the rotation of crops,
— keeping quality is not influenced by the time of harvesting, by the climatic conditions of harvesting or by the character of soil.

The method of harvesting is important because it determines the loss caused by injury. Damaged roots have to be separated from the sound ones in order to reduce spoilage in the storage space. The percentage of injured roots is much higher in mechanical than in manual harvesting.

According to data in the literature the most favourable conditions of the storage of root vegetables are a temperature of 1–4 °C and 90–95% relative humidity (RH) (ZIEGLER & BÖTTCHER, 1966; BESLER, 1970; LINDSAY & NEALE, 1971; CRISP, 1972; HÁJAS, 1972; SIEDEL *et al.*, 1972; LÉPOLD, 1973; SMITH, 1967; ERNEST, 1972). Carrots keep well under the above conditions for 6–8 months (CRISP, 1972).

Standard ISO/TC 34 556 E specifies a storage period of 4–6 months for carrots.

In 1971 through 1972 storage experiments were carried out at the RESEARCH STATION, Fertőd, of the INSTITUTE FOR HORTICULTURAL RESEARCH (HÁJAS, 1972). Carrots were stored in wooden containers and in plastic bags. Part of the roots was washed prior to storage. Some of the containers were covered with plastic film, others were not. Storage was carried out at 1 and 4 °C, resp. The most favourable storage conditions were found to be 1 °C temperature for roots not washed and stored either in polyethylene bags or in wooden containers.

ZHADA and co-workers (1969) stored carrot variety *Chantenay* at 97–98% RH and 0.4–2.0 °C for 160 days. These conditions were achieved by mixing the recirculated air with 1–3% fresh air and passing it over the surface of water. By this method of air conditioning the keeping quality was increased by 10% in relation to direct aeration.

According to STOLL (1971) the storage of carrots (and cellery) in controlled atmosphere is most efficient when the air contains 3% oxygen, 3% CO_2 and 94% N_2. If the CO_2 content of the atmosphere increases above the specified level the spoilage of the vegetables becomes more rapid.

WEICHMANN (1973) found that when carrots are stored in an atmosphere containing more than 1% CO_2 spoilage is increased and a CO_2 content above 5% causes damage to the tissues of celery.

SIEDEL and co-workers (1972) found that an atmosphere containing 5% CO_2 was optimal for the storage of carrots. During an 11-week period spoilage amounted to 13% of which 2% were caused by mildew. In their opinion, in agreement with other authors, it is most important to stabilize the temperature and shorten the cooling period as far as possible.

MARCELLIN (1972) studied a new method of storage. He applied a plastic film for covering, a part of which was replaced by a layer of specific permeability. This part is called the "window" and its size depends on the dimensions of the storage space and on the kind of raw material stored and on the quantity. The size of the plastic film permits of covering 300–600 kg produce

(AC 500 *Rhone–Poulenc*) or 20–50 kg (AC 20 and AC 50 *Rhone–Poulenc)*.

By means of the "window" an atmosphere of 2–3% O_2 and of 3–5% CO_2 is ensured for the storage of apples and pears. For carrots the atmosphere contains 12–14% O_2 and 2% CO_2.

Where the climatic conditions permit it, a possibility of an advantageous and cheap method of storage is available: the storage of root vegetables in wooden cases out of door.

The advantages of this storage method against storage in stacks are: a lower space requirement, centres of spoilage may easily be removed, the channels of ventillation left between blocks of cases permits of regular control. It permits of safe and rhythmical service of the processing line. Loss on storage is minimal. It is shown to be economical by calculations. Efficiency of the method was improved when the vegetables were picked not too early (LÉPOLD, 1973).

The disadvantages of fluctuating storage temperature were investigated with varieties *Flakker* and *Rotherz* (LÉPOLD, 1973). Infection by bacteria and mildew could not be avoided by the most careful preparation of the produce. In order to inhibit propagation of the two most frequent microorganisms *(Centrospora acerina* and *Sclerotinia sclerotiorum)* carrot varieties *Chantenay* and *Amsterdamer Treib* were treated with different plant-protecting agents. The best result was obtained by immersing or soaking the roots in a solution of Benomyl. After a 3-month storage period spoilage of roots not treated was four-fold of that of the treated ones. After 7 months the difference was still three-fold (ERNEST, 1972).

The danger of *Centrospora* infection lies in the fact that this microorganism grows better at low temperature than at higher temperature.

Carrots, celery, cauliflower, parsley and potatoes were stored by PRINCE (1971) at 1 and at 15 °C. The experiments were aimed at clarifying the time of appearance and place of contalmination. It was found that the vegetables arrive from the place of growing in an infected state. Contamination in celery is lighter if seedlings are put out later. This phenomenon seems to be dependent on the weather conditions. The situation is similar with carrots. The author succeded in separating a compound from carrots, which inhibits the growth of *Centrospora.*

An assay was made to inhibit microbial spoilage of carrots and parsley by treatment with ionizing radiations. KISS and co-workers (1972) examined the effect of radiation doses of 5 to 15 krad upon the two vegetables stored at 5 °C. The spoilage of carrots was increased by the treatment.

The aim of the present study was to select the variety most suitable for industrial processing.

1. Materials and methods

1.1. Raw materials

The raw material was obtained from the RESEARCH STATION, Fertőd, of the INSTITUTE FOR HORTICULTURAL RESEARCH.

The conditions of cultivation were as follows. Varieties were grown in a medium-bound clay soil. It received manure in 1972 (30 tons per hectare) and was fertilizer-treated in 1973 (0.4 tons superphosphate, 0.4 tons potassium, 0.15 tons nitrogen per hectare). No irrigation was applied.

The following 6 carrot varieties were studied:

1. *Amsterdamer Treib*
2. *Gonsenheimer*
3. *Nantoise*
4. *Chantenay*
5. *Fertődi vörös*
6. *Flakker (Red giant)*

Many specimens of variety *Gonsenheimer* were cracked. In variety *Amsterdamer Treib* there were also a number of damaged roots.

The varieties were characterized as follows (BECKER *et al.*, 1973):

Amsterdamer Treib carrots [*Daucus carota* L. *ssp. sativus Arcangeli* (Hoffm.)]:

Economic value. Early variety, therefore suitable for forcing under plastic film or glass-house, or early growing in the field. It grows rapidly. Its cultivation period of medium length permits of second sowing (100–120 days from sprouting to picking).

Tied in bundles, it is sold mostly to be consumed fresh. The root is slim, cylindrical. It is of orange-red colour, suitable for exporting. It is a reliable variety of good productivity. Its heart is of the same colour as the outer layer and of medium size and because of its composition it is used for canning. It constitutes about 1% of the total carrot crop in Hungary. It grows well in sandy, light clay.

Morphological characteristics. The surface of the root is slightly ringed. It is of medium size: length 14–16 cm, width 2.5–3 cm. The colour is orange-red, the inside is of the same colour as the surface. The flesh is medium compact.

Variety *Gonsenheimer* [*Daucus carota* L. *ssp. sativus Arcangeli* (Hoffm.)]:

Economic value. Very early. Suitable for growing in hot-house or under plastic film. Recommendable for early cultivation in the field. Grows rapidly. Growth season 75–100 days.

Tied in bundles it is sold mostly to be consumed fresh. Because of its rapid growth it is suitable for second sowing, mainly in gardens. Being of

good colour it may be used for canning. It grows well in sandy soil. It forms about 4% of the carrot crop in Hungary.

Morphological characteristics. The root is of medium length, cylindrical, with a conical end. Average size: length 9–10 cm, width 2.0–2.5 cm. The surface is slightly ringed, of orange-red colour. The middle is of medium size and its colour resembles that of the surface. The quality of the flesh is good.

Variety *Nantoise* [*Daucus carota* L. *ssp. sativus Arcangeli* (Hoffm.)]:

Economic value. A well-known, generally cultivated variety, it forms about 20% of the total carrot crop of Hungary. Because of its short growth period (105–130 days) it is used first of all for early growing in the field. Picked in the autumn it can be consumed fresh and is also suitable for storage during the winter period. Tied in bundles, it is sold mostly for fresh consumption. Because of its even thickness and cylindrical shape it is suitable for mechanical peeling and slicing, lending itself for canning. Its weakness is that the roots incline to crack. It can be best grown on light, medium-bound soil.

Morphological characteristics. The root is of even thickness, cylindrical shape, it has the same thickness both at the shoulders and at the root-body, with a blunt end. It has a medium length of 14–16 cm, its width is 3–4 cm. The surface of the root is slightly ringed, it has an orange-red colour, the peel is thin. This variety can easily be damaged. Its tissue is compact and flexible. It cracks and spoils easily. Its heart is small and of the same colour as the flesh. The leaves are susceptible to diseases.

Variety *Chantenay (Arany, Rotherz)* [*Daucus carota* L. *ssp. sativus Archangeli* (Hoffm.)]:

Economic value. It is grown everywhere in the country. It forms about 25% of the total crop. It is favoured by the canning industry. Because of its stocky shape, smooth surface it is suitable for mechanical peeling and slicing. It may be used for drying. The growth season is of medium length (120–140 days from sprouting to picking). A variety of good productivity. Picked in the summer or in the autumn it may be consumed fresh. Its keeping quality is satisfactory. It gives a good crop on bound soil. Recommendable for mechanical harvesting. It is resistant to diseases and utilized in many ways.

Morphological characteristics. The root has the proper root shape, from the wide shoulder gradually tapering towards the characteristically blunt end. The average size: length of 12–13 cm, width of 5–6 cm. The surface is smooth, of orange-red colour. The colour of the flash is darker, the heart is of medium size.

Variety *Fertődi vörös* [*Daucus carota* L. *ssp. sativus Arcangeli* (Hoffm.)]:

Economic value. Suitable for processing. The growth period is long (200–220 days from sprouting to harvesting). Its yield is much higher than that of the original variety: *Bauers Kieler Rote*. It has an attractive shape and it is rich in carotene and of a high solids content. It is more satisfactory from

the consumer's aspect than other varieties generally grown at present. It is suitable for large-scale cultivation and mechanical harvesting. It also may be grown in the garden because of its good keeping quality.

Morphological characteristics. The root has slightly sloping shoulder, it is uniformly tapering downwards and ends in a point. Its surface is slightly ringed, of a vivid red colour. The heart is of medium size, of the same colour as the flesh, or somewhat darker.

Variety *Flakker (Red giant)* [*Daucus carota* L. *ssp. sativus Arcangeli* (Hoffm.)]:

Economic value: It forms about 20% of the total crop. It is of a long growth period (160–180 days from sprouting to harvesting) and of high productivity.

It is grown mainly for winter-storage, but it may be used in the canning industry due to its good shape. However, because of its light colour, medium solids and carotene contents it is not favoured. Its most important quality is its unfailing yield. It is a variety resistant to diseases.

1.2. Storage

The roots were selected and stored in cases. The temperature of the storage space was 9–11 °C and the relative humidity 70–85%.

1.3. Spoilage

Every root, suffering change on one sixth of its surface, caused by mildew or rotting, was considered spoiled. The weight of the spoiled pieces was established and expressed as percentage of the initial weight.

1.4. Weighing

Each case was weighed on 0 day, after 5 and 9 weeks, resp. Weight loss is given as percentage of the initial weight. Each case contained 15–20 kg of carrots and depending on the quantity in one case, 4–8 cases were weighed.

1.5. Total solids content

Of each variety five pieces were grated and three parallel measurements were made of the mixed average of the gratings. The sample of known initial weight was dried to constant weight at 105 °C.

1.6. Texture

The consistency was measured with a *Texturometer* (ZENKEN Co., Japan) by the method according to KOVÁCS and VAS (1969). Twelve-cm long slices were cut from the carrot and used for measurement in 5 parallels ($N = 5$).

1.7. Sensory evaluation

The varieties were tested in the raw state and cooked. The roots were washed, wiped and cut in half. The colour, smell, consistency and withering were scored on a hedonic scale of 5 points, point 1 corresponding to very poor, point 5 to excellent.

For cooking, the roots were peeled, cut into pieces and cooked in water containing 1.5% NaCl (w/v) for 20 min. Proportion of brine to carrots 2:1. During the organoleptic test the samples were kept in the brine. Samples were scored for colour, smell, taste, degree of doneness and stringiness, on a 5-point scale, point 1 corresponding to the poorest, point 5 to the best sample. Scores were ranked and evaluated according to KRAMER (1960).

2. Results

2.1. Liability to deterioration

On examining the varieties for liability to deterioration variety *Chantenay* was found to suffer the lowest weight loss during the 11-week storage period (Table 1, Fig. 1).

Variety *Fertődi vörös* was stored indiscriminately and selected. Spoilage in the indiscriminately stored sample was substantially higher than in the selected sample. This variety was also classified according to size, since originally

Table 1

Keeping quality of different carrot varieties based on the time required to reach 5, 10 and 20% spoilage and weight loss, resp.

Variety	N	Time required to reach					
		5	10	20	5	10	20
		% spoilage			% weight loss		
Early varieties							
Amsterdamer Treib*	8	4.8	10.0		2.5	5.2	
Gonsenheimer*	9	3.1	6.5		2.7	5.6	
Nantoise*	5	7.3	<10.0		1.7	3.5	10.5
Varieties for industrial use and storage							
Chantenay*	5	<5.0	<5.0		4.6	7.9	
Flakker*	3	6.9	10.0		2.1	4.3	
Fertődi vörös							
sorted, long	3	2.6	5.6		2.1	4.3	
sorted, short	3	1.9	3.8	10.0	2.8	5.7	
not sorted	3	0.6	1.2	3.6	2.1	4.5	10.6

20% spoilage or weight loss was reached only by a few samples within the 11-week storage period

N = number of cases (boxes)

* = selected sample

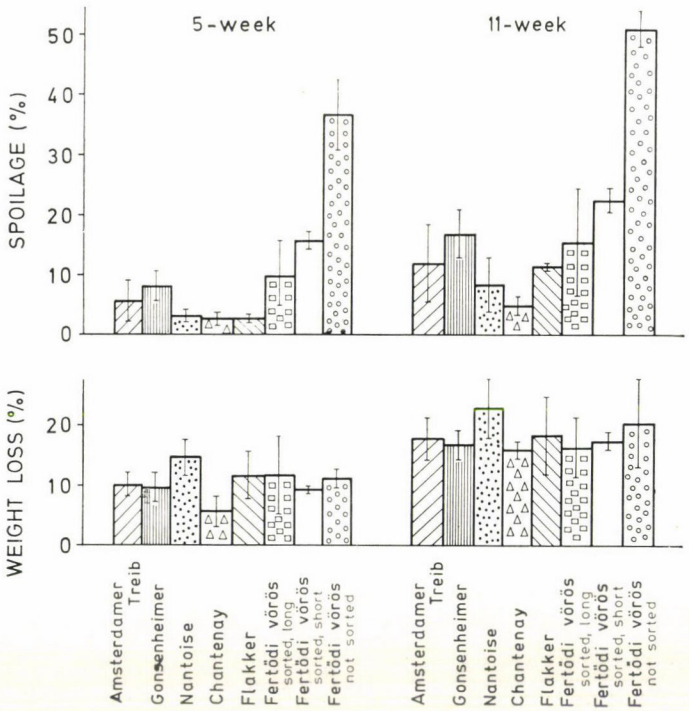

Fig. 1. Weight loss and spoilage of different carrot varieties as a function of storage period (at 9–11 °C and 70–85% RH). I = standard deviation

it was extremely heterogeneous. A difference was observed in the spoilage of roots above and below 10 cm length, the longer ones being more resistant to spoilage.

Five per cent spoilage was reached first by variety *Fertődi vörös* (in 2.6 weeks) and last by *Chantenay* (in 5 weeks). Results as regards 10% spoilage were similar. Variety *Flakker* was of a good keeping quality, too.

2.2. Liability to weight loss

The weight loss of carrot varieties stored at 9–11 °C and 70–85% RH for 11 weeks varied between 15 and 20% (Fig. 1).

The lowest weight loss was observed with variety *Chantenay* and this was 15.4%. The results of the t test of significance of differences between the average values of weight losses as measured after an 11-week storage period, are shown in Table 2.

The highest weight loss was found with variety *Nantoise*. This variety was significantly worse than variety *Amsterdamer Treib* ($P \geq 95\%$), variety *Gonsenheimer* ($P \geq 99\%$) and variety *Chantenay* ($P \geq 95\%$).

Table 2

Test of significance of differences between weight losses experienced with different carrot varieties. t test after 11 weeks of storage at 9–11 °C and 70–85% RH

Variety	N \ N	Gonsenheimer	Nantoise	Chantenay	Flakker	Fertődi vörös
		9	5	5	3	3
Amsterdamer Treib	8	0.5564***	2.2748*	1.0370	0.2076	0.1882
Gonsenheimer	9		3.2126**	0.7925	0.5416	0.6048
Nantoise	5			3.1268*	1.0374	1.5786
Chantenay	5				0.7081	0.8331
Flakker	3					0.0583

N = number of cases (boxes)
* = samples differ at 95% probability level
** = samples differ at 99% probability level
*** = samples differ at 99.9% probability level

2.3. *Changes in the solids content*

The solids content of the carrot varieties varied between 9.6 and 13.5% at the start of storage. The variety of highest solids content was *Fertődi vörös*.

The solids content of all samples increased but slightly during storage (Table 3).

Table 3

Solids content of the different carrot varieties as a function of storage time (at 9–11 °C and 70–85% RH)

Variety	Storage period (week)										
	0			5			11			5	11
	Solids content %									Corrected values	
	n	$\bar{x}$	s	n	$\bar{x}$	s	n	$\bar{x}$	s	$\bar{x}$	$\bar{x}$
Early varieties											
Amsterdamer Treib	3	11.91	2.290	3	10.16	0.319	3	10.55	0.112	9.17	8.72
Gonsenheimer	3	11.59	0.917	3	10.12	0.127	3	11.77	0.834	9.18	9.83
Nantoise	3	9.61	0.134	3	11.26	0.556	3	12.19	0.455	9.65	9.40
Varieties for industrial use and storage											
Chantenay	3	11.59	0.179	3	11.97	0.558	3	12.27	0.143	11.31	10.37
Flakker	3	10.75	0.073	3	10.97	0.268	3	12.24	0.245	9.71	10.04
Fertődi vörös	3	13.53	0.091	3	13.13	0.306	3	14.61	0.168	11.64	11.66

N = number of parallel measurements
$\bar{x}$ = average
s = standard deviation

On comparing the values of solids content to the relevant weight loss values a slight decrease of the solids contents could be observed.

2.4. Changes in texture

Texture values changed during storage as shown in Table 4 and Fig. 2.

The correlation between weight loss and change of texture was investigated (Fig. 3).

Table 4

Texturometer values of different carrot varieties as a function of storage period (9–11 °C, 80–85% RH)

Variety	0 weeks			5 weeks			11 weeks			Change during 11 weeks (%)	Rank
	N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s		
Amsterdamer Treib	3	187.0	23.67	5	194.3	23.93	5	142.0*	11.04	24.1	1
Gonsenheimer	3	199.5	9.60	5	185.6	17.46	5	150.0***	11.55	24.8	2
Nantoise	3	199.8	21.61	5	185.0	27.18	5	126.7***	11.98	36.6	4
Chantenay	3	221.5	8.26	5	177.3	13.74	5	139.8***	24.33	36.9	5
Flakker	3	202.5	16.20	5	187.7	19.27	5	130.7**	20.28	35.5	3
Fertődi vörös	3	231.7	5.74	5	216.5	7.61	5	142.5***	14.31	38.5	6

N = number of parallel measurements
$\bar{x}$ = average
s = standard deviation
* = difference is significant between data at 0 and 11 weeks ($P \geq 95\%$)
** = difference is highly significant between data at 0 and 11 weeks ($P \geq 99\%$)
*** = difference is very highly significant between data at 0 and 11 weeks ($P \geq 99.9\%$)
Between data measured on the same day, differences were non-significant ($P < 95\%$)

A correlation coefficient of $r = 0.779$ was found between the weight loss and texture of each variety. On the basis of the r^2 value it may be established that weight loss is to 61% responsible for the changes in texture.

2.5. Changes in sensory value

The different carrot varieties were evaluated in the raw state and cooked, on 0 day and after 5 and 11-week storage, resp.

2.5.1. Raw samples. In the raw state the colour of variety *Gonsenheimer* was significantly lower, that of *Fertődi vörös* significantly higher than the colour of the other varieties. As regards general appearance, variety *Chantenay* scored highest.

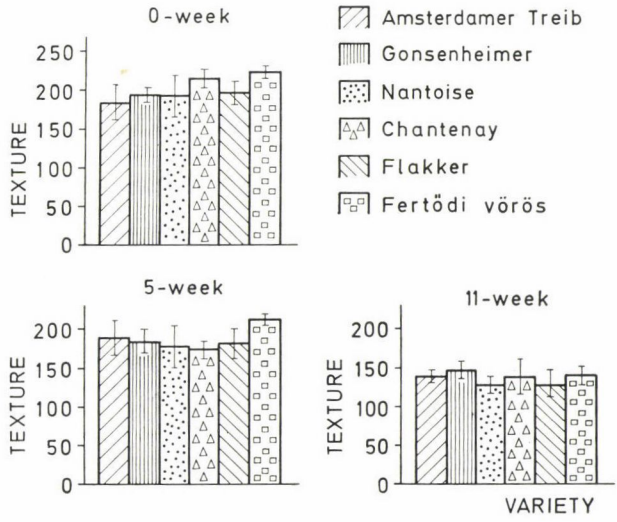

Fig. 2. Texture changes of different carrot varieties as a function of storage time. I = standard deviation

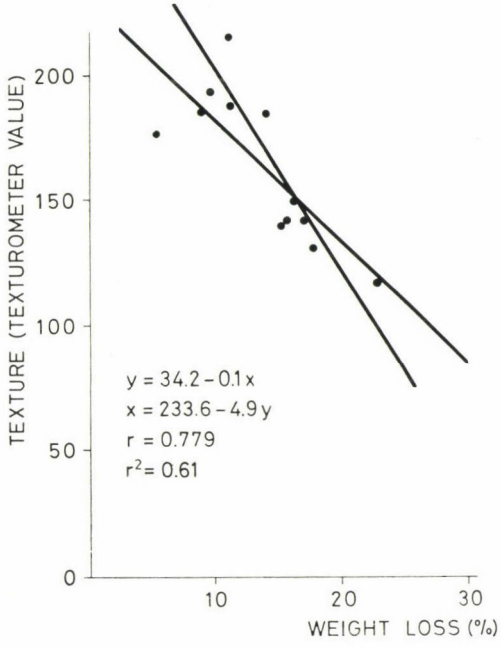

Fig. 3. Test of correlation of weight loss and change of texture of different carrot varieties

After 5 and 11 weeks storage the difference in colour, smell and texture between varieties was not significant (Table 5).

Table 5

Comparison of the sensory values of different carrot varieties at different points of time in the raw state and cooked

Storage period (week)	Variety	Raw			Cooked			
		colour	smell	texture	colour	smell	taste	doneness
	Amsterdamer Treib	–	–	–	–	–	–	–
	Gonsenheimer	(*)	–	–	–	–	–	–
	Nantoise	–	–	–	–	–	–	–
0	Chantenay	–	–	**	(*)	–	–	–
	Flakker	–	–	–	–	–	–	–
	Fertődi vörös	**	–	–	**	–	**	–
	Amsterdamer Treib	–	–	–	*	–	–	(*)
	Gonsenheimer	–	–	–	–	–	–	–
	Nantoise	–	–	–	–	–	–	–
5	Chantenay	–	–	–	(**)	–	–	–
	Flakker	–	–	–	–	–	–	–
	Fertődi vörös	–	–	–	–	–	–	–
	Amsterdamer Treib	–	–	–	*	–	–	–
	Gonsenheimer	–	–	–	–	–	–	–
	Nantoise	–	–	–	–	–	–	–
11	Chantenay	–	–	–	–	–	–	–
	Flakker	–	–	–	–	–	–	–
	Fertődi vörös	–	–	–	–	–	–	–

Symbols used:
The property of the variety indicated
– = does not differ significantly from that of the others ($P \geq 95\%$)
* = is significantly better than the rest ($P \geq 95\%$)
(*) = is significantly worse than the rest ($P \geq 95\%$)
** = is highly significantly better than the rest ($P \geq 99\%$)
(**) = is highly significantly worse than the rest ($P \geq 99\%$)

2.5.2. Cooked samples. On 0 day *Fertődi vörös* had the best ($P \geq 99\%$), *Flakker* ($P \geq 99\%$) and *Chantenay* ($P \geq 95\%$) the palest colour.

The taste of variety *Fertődi vörös* was significantly better ($P \geq 99\%$) than that of the other samples. In other properties differences were not significant.

After 5 weeks the colour of variety *Chantenay* was significantly lower ($P \geq 99\%$), that of variety *Amsterdamer Treib* significantly better ($P \geq 95\%$) than that of the other varieties.

The differences in smell, taste and degree of doneness were not significant between varieties, except for variety *Amsterdamer Treib*, which was worse than the others at the probability level of 95%.

After 11 weeks the colour of variety *Amsterdamer Treib* was significantly better ($P \geq 95\%$) than that of the rest. The differences in other properties were not significant.

2.6. Ranking of varieties

In Table 6 varieties are ranked according to their liability to spoilage, weight loss and changes in texture during storage.

Table 6

Different carrot varieties ranked according to their keeping quality

Properties tested	Variety					
	Amsterdamer Treib	Gonsenheimer	Nantoise	Chantenay	Flakker	Fertődi vörös
	Rank numbers					
Liability to spoilage (10 %)	4	5	1.5	1.5	3	6
Weight loss (10 %)	3	2	6	1	4	5
Change of texture	1	2	4	5	3	6
Rank sum	8	9	11.5	7.5	10	17
Percentage of total crop in Hungary (BECKER *et al*, 1973)	1	4	20	25	20	no data
Yield, t ha^{-1}	180	170	185	280	360	350
Ranking according to yield	5	6	4	3	1	2
Rank sum based on the properties tested and on yield	13	15	15.5	10.5	11	19

— The best variety has the smallest rank sum, the worse is the one with the highest rank sum
— Sensory qualities and solids content were not shown, because for lack of differences, they could not be evaluated
— Liability to deterioration was judged by the time needed to reach 10% spoilage, weight loss by the time of reaching 10% loss. Changes in texture represent the decrease in *Texturometer* values as a percentage of the inital value.

There was no significant difference in sensory values and solid contents after the 11-week storage period, therefore these data were omitted.

On the basis of results, the order of varieties according to their keeping quality is as follows:

Chantenay, Amsterdamer Treib, Gonsenheimer, Flakker, Nantoise, Fertődi vörös, sorted, long.

Varieties ranked according to their technological properties, also taking into account their yield, gave the following sequence:

Chantenay
Flakker
Amsterdamer Treib
Gonsenheimer
Nantoise
Fertődi vörös

3. Conclusions

Of the 6 carrot varieties studied 3 were early type *(Gonsenheimer, Amsterdamer Treib, Nantoise)* the other 3 suitable for processing and storage *(Chantenay, Flakker, Fertődi vörös)*. *Fertődi vörös* has been recently accepted as a variety. It was improved in Hungary. To the knowledge of the authors this variety has not been studied for keeping quality before.

In most cases the roots were selected. *Fertődi vörös* was also classified into roots below 10 cm length and above this size. A part of the longer roots were selected, the rest was stored without sorting.

Judged by liability to spoilage, weight loss and texture, veriety *Chantenay* seemed to be the most suitable for storage, while *Fertődi vörös* had the lowest keeping quality.

Varieties *Amsterdamer Treib*, *Gonsenheimer* and *Nantoise* were also found suitable for storage.

Toma (1973) in his storage experiments in cases kept out of door, found *Flakker* and *Rotherz* (a *Chantenay*-type variety) also suitable for storage.

The highest solids content was found in variety *Fertődi vörös* (13.5%), the lowest in variety *Nantoise* (9.6%). The solids content changed very little or not at all during the 11-week storage period.

Hájas (1972) did not use carrots true to variety in her experiment. She found the solids content to increase till February, thereafter a decrease was observed. Changes were insignificant when samples were stored in polyethylene bags.

The difference in texture of every variety was found significant after 11 weeks as compared to the initial texture on 0 day. The lowest difference was

measured with varieties *Amsterdamer Treib* (24.1%), and *Gonsenheimer* (24.8%).

HOWARD and HEINZ (1970) carried out similar experiments. These authors studied the compressibility and resistance to cutting in carrots (*Instron Universal Machine*, Model TM apparatus). They carried out the measurements in the middle of the root because according to their experience changes were most extensive here. They found a close correlation between objective data of compressibility and elasticity, on the one hand and results of sensory evalution, on the other.

The correlation was, however, not close between data of resistance to cutting and sensory values.

When using the *Texturometer* the most acceptable results were obtained with the bronze-tooth fitting. Originally it was intended to use the cylindrical plexi-glass fitting. However because of the hardness of carrots results thus obtained were not acceptable. It is hoped that the results obtained with the bronze-tooth fitting characterise adequately the texture of the samples.

*

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NEW EXPERIMENTAL AND MATHEMATICAL METHODS CONCERNING GLASS STRENGTH PROBLEMS IN PALLET STACKS OF CANNING JARS

PART I. — REVIEW OF THE LITERATURE AND THEORETICAL CONCEPTS

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Two basic aspects of accounting for the breaking strength of glass jars are described. The concept of the step-wise breaking process is introduced with respect to layers of glass jars on pallets and a modified polynomial distribution for the probability of the occurrence of a specified step-wise breaking process in case of layers containing finite number of jars is derived. A method for the calculation of the average breaking ratio (percentage) is described by accounting for the step-wise breaking process when large numbers of layers are manipulated. A method of calculation is presented for the determination of the probability that during the step-wise breaking process not more of jars will break than the number predetermined by safety aspects. A computerized calculation is suggested for the solution to this problem. The concept of the step-wise breaking process is generalized for glass layers containing great number of jars, theoretically the number approaching to infinity. The concepts of critical primary load and critical average load are introduced for the above layers and both the calculation method and the graphic solution are given for the determination of these values and of the average breaking ratio.

Transport and storage of canning jars on pallets has great economic advantages. Pallets were introduced in Hungary in 1963 and since then many papers have appeared on the pertaining technical, economic and organizational problems. The list of these publications can be found in the manuscripts* and published papers of KÖRMENDY (1975) and KÖRMENDY-BALTHAZÁR (1975a, 1975b, 1976). From this list only the publications with direct bearing on the present work will be mentioned here.

VARGA and KARAI (1968) measured the load acting on single 5/4 glass jars inside the stack and drew conclusions from the results for the necessary technical improvements. They used a special measuring cell of the same dimensions and shape as the 5/4 glass jar, thus they could replace any glass jar within the stack by the cell.

FERENCZY and KUNOS (1973) performed strength tests on various types of glass jars both filled with foodstuff and empty. One part of the tests was carried out on single jars by applying axial load, and others on the complete stack. The results have been made available in the form of a mimeographed manuscript by the RESEARCH INSTITUTE OF THE CANNING AND PAPRIKA PROCESSING INDUSTRY, Budapest to the enterprises under the authority of the TRUST OF THE ENTERPRISES OF THE CANNING INDUSTRY, Budapest.

* The manuscripts are available in mimeographed form in the RESEARCH INSTITUTE OF THE CANNING AND PAPRIKA INDUSTRY, Budapest.

Because of the limited number of jars used in the experiments and certain so far insufficiently elaborated methodological problems the work of these authors was considered as preliminary experiment. However, in the present study and in our previous works we have more than once relied on their experimental data.

We were unable to find any useful published data referring to actual loads under the conditions of stacks in the pertaining foreign literature which were in connnection with our work. The data we have found referred to the loadability of the pallets, to the height of the stack or to various practical suggestions.

A number of papers and books deal with the problems of the strength of glass as a structural material, of which we mention the books of LACOURSE (1972) and KERKHOF (1970). The aim of extensive research is to produce a highly suitable material for technical purposes. The glass products have, as generally known, a high compression strength and a low resistance to tensile stresses. The latter is primarily determined by the state of the glass surface. Glass begins to break starting practically always from the surface and only under the effect of tensile stress. The reason for the low tensile strength can be attributed to the small, often microscopic injuries on the glass surface. Specimens etched with fluoric acid to obtain a smooth surface and coated with a protective layer have often 50 times higher tensile strengths than the original speciemens.

The composition of the medium in contact with the glass surface might have also a significant effect on its strength.

The rate of load increase must also be taken into consideration. When the load is rapidly increased glass might appear to have a higher strength. Opinions agree insofar as even under well-defined experimental conditions the variance of experimental data is very large concerning the strength characteristics of glass. According to SHAND (1958) the strength values fail to correspond to the normal distribution.

Some data have been published on the strength of glass containers. PHILLIPS (1960) cites certain results according to which in case of beer bottles the highest tensile stress occurs where the cylindrical part is joined to the bottom when the bottle is subject to internal pressure. According to MOODY (1963) the internal pressure is raised every 60 s when measuring the strength of glass bottles at room temperature. He calculated the highest tensile stress inside the cylindrical part, but implicitly neglected the bending moment, and assumed that the breakage starts at the maximum tensile stress. MOODY also claims that when axial loads are applied, the experiments must be planned according to the same principles as in the case of internal pressure. HOUGEN (1970) described various types of fractures which he illustrated on figures.

There are also a few standards on the loading of glass vessels by internal pressure (ASTM Designation C 147; DIN 52 320) which specify the rate of pressure increase to be applied for achieving the various pressure levels and also the length of time for keeping the glass under pressure.

1. Basic concepts and general considerations*

Figure 1 shows as an example a column consisting of two stacks on top of one another. Figure 2 is the sketch of an experimental device for breaking each jar separately. Both figures serve at the same time to illustrate terms used in this paper.

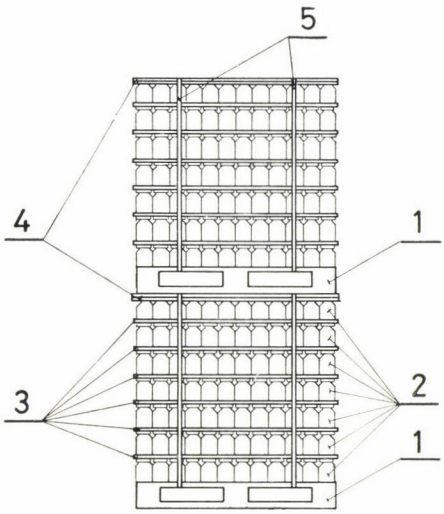

Fig. 1. Column consisting of two stacks on top of each other. **1**: pallet; **2**: layers of glass jars; **3**: load distributing plates; **4**: clamping and load distributing top plates; **5**: fastening straps

We shall deal first of all with canning jars filled with foodstuffs in whose interior there is a smaller or greater depression (vacuum). The described principles and methods will, of course, be applicable also to empty jars and other glass vessels, provided loads are acting similar to those occurring in the case of canning jars.

The canning industry uses such great number of glass jars, filled with foodstuff and regarding a specified type of jar, that their entirety can be taken as a set of an infinite number of elements, this set will be designated by the symbol $[U]$. To each element of the set, that is to every jar, a given vacuum (p_i)

* A list of symbols is attached to Part II of this article

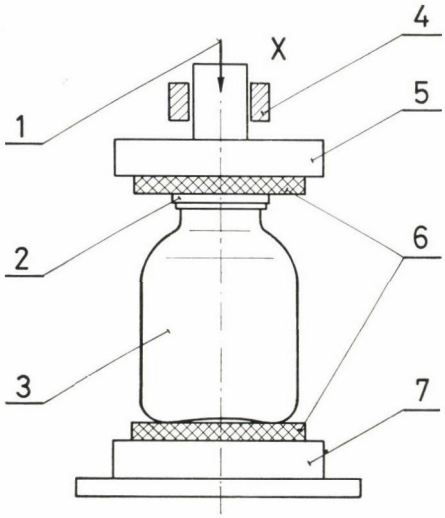

Fig. 2. Sketch illustrating the principle of the test equipment for the individual breaking of jars. **1**: load or breaking force; **2**: cap of the jar; **3**: canning jar; **4**: shaft-locating bearing; **5**: pressure plate; **6**: cushioned pads; **7**: supporting plate

is assigned whose value varies practically between 0 and 1 kp cm^{-2}. In the same way a breaking experiment can be assigned to every jar whose result furnishes the breaking force x_i. When using the concept of breaking strength we mean always the breaking force. The breaking force x_i and the vacuum p_i are interpreted as probability variables.

The cumulative distribution function $\Psi(p)$ gives the ratio of jars in which the vacuum is lower than the value of p. The cumulative distribution function $\Phi(x)$ indicates the ratio of jars which breaks under the load x when the vacuum in the individual jars has a certain value p_i between the formerly discussed limits. Thus, in course of the breaking experiment the original value of the vacuum in the jars filled with foodstuff is not changed.

When the breaking experiment is performed by placing the jar under a definite vacuum, the conditional cumulative distribution function $\Phi(x|p = \text{constant})$ will be interpreted on the set $[U]$ by replacing the constant with the definite value of the vacuum. For instance, in the case $\Phi(x|p = 0)$ there is no vacuum in the jar subjected to the breaking test.

Strictly speaking the definition of the breaking force depends upon the apparatus used for the test and upon the experimental conditions. Figure 2 explains the basic principle of the breaking test. By means of this arrangement an approximation of either the cumulative distribution function $\Phi(x)$ or $\Phi(x|p = 0)$ can be obtained, since either the original vacuum in the jar is left unchanged or reduced to zero by opening the lid.

The character of the cumulative distribution function $\Phi(x)$ and the pertaining probability density function $\varphi(x)$ are illustrated in Fig. 3. The Figure was plotted from the data published in the manuscript of FERENCZY and KUNOS (1973). The curve in Fig. 3 represents the set of course only approximately.

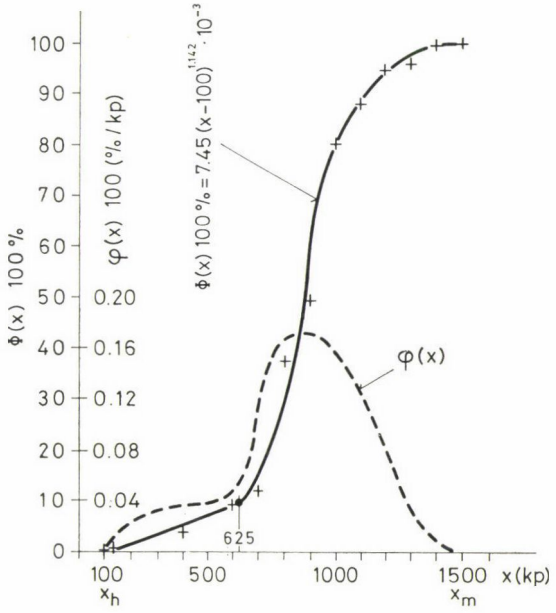

Fig. 3. Example illustrating the cumulative distribution function $\Phi(x)$ and density function $\varphi(x)$ obtained from the individual breaking of the jars

In agreement with the symbols in Fig. 3 it is evident that there is a force x_h under which none of the jars will break and also a force of finite magnitude x_m at which and above which all jars of the set will break. The force x_h is identical with the minimum breaking strength and x_m with the maximum breaking strength and the value of the first might be even zero. It follows that though theoretically $\Phi(x)$ can not be a normal cumulative distribution function, it is possible that the middle section of the function should be approached by normal distribution.

It seems justified to assume from the data obtained so far that the $\Phi(x)$ cumulative distribution functions of the various types of canning jars increase in the strict sense monotonously between x_h and x_m, *i. e.* no horizontal sections are present in this range.

In principle it is possible that the set $[U]$ consists of a mixture of two or more types of jars for which the ranges between the minimum and maximum breaking strength do not overlap. In this case the value of the cumulative

distribution function is constant in the zones between the limits of the individual ranges. This case is illustrated in Fig. 4. It appears clearly from the figure that none of the glass jars break in the range between x_{m1} and x_{h2}, for all jars of type 1 are already broken at x_{m1}, while the jars of type 2 do not break when the force is equal or lower than x_{h2}.

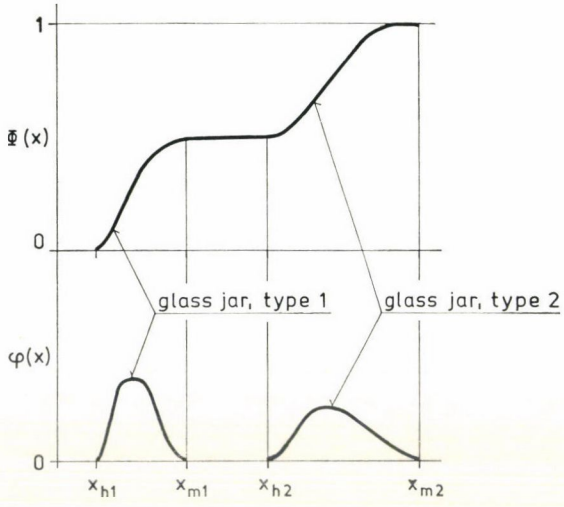

Fig. 4. Cumulative distribution and probability density function of a set consisting of the mixture of two types of glass jars. There is no overlapping of the ranges limited by the minimum and maximum breaking strengths

For the experimental determination of the cumulative distribution function $\Phi(x)$ the general rules of sampling are of course valid.

The breaking strength of glass jars will be considered from two aspects in order to have a basis for theoretical considerations and for the planning of the experiments.

One aspect is to keep the average breaking ratio in loaded stacks below a specified value which necessitates a certain amount of organization and registration whose details will be given in Part II. Because of this aspect the breaking tests must be performed with respect to the range of loads occurring under the conditions of the storing of stacks.

VARGA and KARAI (1968) were the first to call attention to the aspect which involves the simultaneous breaking of several jars which might lead to the collapse or overturning of the stacks and might therefore be the source of some major damage or accident, though the probability of such event be comparatively low. Because of this aspect certain methods of probability calculation and special experimental procedures had to be introduced as will be shown in para. 2.5 and in Part II.

2. Analysis of loads acting in the stacks and the step-wise breaking process

A specified layer of the stack is subject — as shown by the example in Fig. 1 — to the load due to the weight of the charge above it. It has been agreed to include into this load the own weight of the layer, too.

This load differs from one layer to the other and decreases from the bottom towards the top.

The overall load on a layer is divided among the jars constituting the layer. If suitable load distribution plates are fitted between the layers the load per one jar will only slightly differ from the mean load on the jars.

It is an important fact that the cessation of the load-bearing capacity of the jars broken by the load in the layer implies a greater load on the remaining jars leading to more breakage and thereby to greater loads again and more breakage till the process of breaking stops or all the jars break. This phenomenon will be called the step-wise breaking process or simply step-wise breaking.

The step-wise breaking process can no doubt be described by means of more than one approximation. We believe the method chosen by us to be relatively simple and ready for further development with increasing number of experimental data.

2.1. Analysis of the process of step-wise breaking

In the mathematical description of the step-wise breaking process a simplification is introduced by assuming that in a layer the load on every jar is the same. This load is equal, of course, to the average load which is the total load on the layer divided by the number of jars in the layer. If some of the jars are broken in the layer, the total load on the layer will again be divided uniformly among the remaining undamaged jars.

Let us consider a layer of jars which initially contained N jars, and load the layer with a force so that the average load per jar shall be x_0 kp and assume that this causes the breaking of m_0 jars. In this case the average load on the remaining jars will rise to

$$x_1 = x_0 \frac{N}{N - m_0}. \tag{1}$$

If x_1 causes the breaking of another m_1 jars then the average load will again be higher, namely

$$x_2 = x_0 \frac{N}{N - (m_0 + m_1)} \tag{2}$$

and so on till the process stops or all jars are broken. It may happen that the primary load x_0 does not break a single jar or that this load is enough to break all jars.

The process of step-wise breaking can be illustrated very clearly by graphic means, as shown in Fig. 5. This shows partly a step-wise breaking-process which stops at stage 3 and partly a process in which all jars are broken

The horizontal axis in Fig. 5 shows the average load on a layer consis t

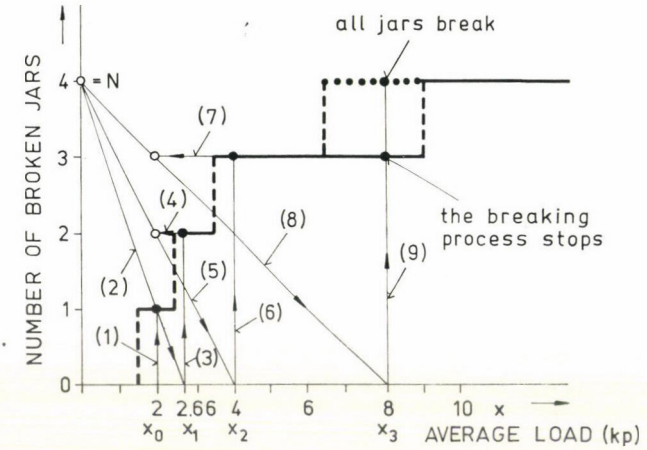

Fig. 5. Illustration of the step-wise breaking process

ing of $N = 4$ jars, while on the vertical axis the number of jars broken by the load have been plotted. In this way a step-wise increasing curve with horizontal sections (thick lines) have been obtained. The horizontal sections are connected by dashed vertical lines at the loads corresponding to the breaking strength of glass. Thus, the breaking strengths of the glass jars are in increasing order: 1.5, 2.5, 3.5 and 9 kp.

Breaking proceeds as follows: at the primary load of $x_0 = 2$ kp the jar with a breaking strength of 1.5 kp breaks and the average load on the remaining jars will be:

$$x_1 = 2\frac{4}{4-1} = 2\frac{4}{3} = 2.66 \text{ kp.}$$

This load $x_1 = 2.66$ kp will break the jar with the breaking strength of 2.5 kp and the average load on the remaining two jars will be

$$x_2 = 2\frac{4}{4-2} = 2\frac{4}{2} = 4 \text{ kp.}$$

A load of 4 kp is already enough to break the jar with a breaking strength of 3.5 kp and the average load will then be

$$x_3 = 2\frac{4}{4-3} = 2\frac{4}{1} = 8 \text{ kp}.$$

At this stage the breaking process stops, since the breaking strength of the last jar is 9 kp, thus being greater than the load of 8 kp and so the last jar will not break. The thin lines with arrows in the figure whose sections are marked by (1), (2) . . . (9) illustrate the constructing of the breaking process in the order of increasing figures. This construction was achieved by simple geometrical considerations such as the theorem of proportional triangles, details being omitted. It appears from the construction that the breaking process stops when the vertical line pertaining to the higher load intersects the same line, representing the number of broken jars, which was intersected before by the vertical line pertaining to the previous load. The dotted horizontal line illustrates the case when the strongest jar breaks at 7 kp and thus will break in the last step since $x_3 = 8$ kp is greater than 7 kp. It appears from the figure that the breaking process will not stop when the vertical line pertaining to the higher load meets one of the horizontal lines one or more steps higher (in the above example the dotted horizontal).

2.2. Probability of the occurrence of a step-wise breaking process

Next we shall investigate the probability of the occurrence of a given step-wise breaking process. For this we have to know first of all the cumulative distribution function $\Phi(x)$ of the breaking strength of the set $[U]$ pertaining to the layer consisting of N randomly chosen jars. Let us consider the case when at the primary load x_0 (step 0) m_0 jars break, the load x_1 breaks m_1 jars and so on.

In the last step load x_r breaks m_r jars. If the value of m_r is zero no jar will break in the last step and the process stops. If m_r is not zero all jars which did not break in the previous steps will now break, so that all N jars will be broken.

Let us give the symbol M to the number of all broken jars. When the breaking process stops, that is $m_r = 0$, then

$$m_0 + m_1 + \ldots + m_j + \ldots + m_{r-1} = M \tag{3}$$

and the number of unbroken jars will be $N-M$.

If the breaking process does not stop, then $N-M = 0$ and

$$m_0 + m_1 + \ldots + m_j + \ldots + m_{r-1} + m_r = M = N. \tag{4}$$

We claim that the probability of the occurrence of any specified variety of a step-wise breaking process can be calculated from the following formula:

$$P = \frac{N!}{m_0!m_1!\ldots m_j!\ldots m_{1r-1}!m_r!\,(N-M)!} \cdot \Phi(x_0)^{m_0} \cdot [\Phi(x_1) - \Phi(x_0)]^{m_1} \ldots$$
$$\ldots [\Phi(x_j) - \Phi(x_{j-1})]^{m_j} \ldots [\Phi(x_{r-1}) - \Phi(x_{r-2})]^{m_{r-1}} \cdot [\Phi(x_r) - \Phi(x_{r-1})]^{m_r} \cdot$$
$$[1 - \Phi(x_r)]^{N-M}. \tag{5}$$

In Eq. (5) the load x_j in step j is determined from the equation

$$x_j = x_0 \frac{N}{N - (m_0 + m_1 + \ldots + m_{j-1})} \tag{6}$$

in agreement with para. 2.1.

If the breaking process interrupts $m_r = 0$ and $N > M$. In this case the value of the factor

$$[\Phi(x_r) - \Phi(x_{r-1})]^{m_r} = [\Phi(x_r) - \Phi(x_{r-1})]^0$$
will be 1 and $m_r! = 0! = 1$.

If the breaking process does not stop, then $m_r \neq 0$, $M = N$ and $[1-\Phi(x_r)]^{N-M} = [1-\Phi(x_r)]^0$ will be 1, and $(N-M)! = 0! = 1$. Among the above factors the indeterminate form 0^0 might also occur and this must be interpreted as 1, since the meaning implies that an impossible event does not occur; the latter statement, being certain, has the probability value of 1.

For the case when the breaking process stops, the verification of Eq. (5) is as follows: The randomly chosen N jars in the layer can be considered as a sample of N elements taken from a set of infinite number of elements. Of these m_0 break at the primary load x_0 with a probability of $\Phi(x_0)^{m_0}$, m_1 jars break between the loads x_1 and x_0 with a probability of $[\Phi(x_1) - \Phi(x_0)]^{m_1}$. Between the loads x_j and x_{j-1} m_j jars will break with a probability of $[\Phi(x_j) - \Phi(x_{j-1})]^{m_j}$ and so on. At the end $N-M$ unbroken jars remain which would break between

$$x_r = x_0 \frac{N}{N-M} \quad \text{and} \quad x_m$$

with a probability of $[1-\Phi(x_r)]^{N-M}$ since the value $\Phi(x_m) = 1$ belongs to x_m (see Figs. 3 and 6).

Since the breaking strengths of the sample elements are independent probability variables, the resultant probability is the product of the probabilities presented above multiplied by the factor consisting of the factorials at the beginning of the equation. The term made up of the factorials gives the number of permutations for replicated items.

Equation (5) might also be obtained by starting instead of a set of jars consisting of infinite number of elements, from a set made up of a finite number (U) of elements from which M_j jars break between the loads x_j and x_{j-1}. Let us give the symbol L to the number of elements whose breaking strength is between x_h and x_r. Then, according to the rules of sampling without replacement the formula

$$P = \frac{\binom{M_0}{m_0}\binom{M_1}{m_1}\cdots\binom{M_j}{m_j}\cdots\binom{M_{r-1}}{m_{r-1}}\binom{U-L}{N-M}}{\binom{U}{N}} \tag{7}$$

expresses the probability that a sample of N elements is selected in the given composition. From Eq. (7) we arrive at Eq. (5) by increasing the value of U beyond all limits. Eq. (5) shows great resemblance to a polynomial distribution. In fact, in many respects there is no difference between our deductions and those known for polynomial distribution (PRÉKOPA, 1962; RÉNYI, 1966).

It stands, however, to reason that despite all formal resemblance Eq. (5) is not a polynomial distribution, since the values in the expression

$$[\Phi(x_1) - \Phi(x_0)], \ldots, [\Phi(x_j) - \Phi(x_{j-1})], \ldots$$

are not constant as the corresponding probability values in polynomial distribution, but depend upon the number of jars broken in each step. For this reason we shall call Eq. (5) a modified polynomial distribution, but wherever justified we shall make use of the statements and relations worked out for polynomial distributions.

The earlier mentioned verification of Eq. (5) might be performed reasonably in the same way for the case when all jars are broken.

Next, we shall quote a numerical example to illustrate the aforesaid. Let us calculate the probabilities of both breaking processes described in para. 2.1 and illustrated in Fig. 5 when the jars have been taken from a set having a cumulative distribution function of $\Phi(x) = x^2/100$.

The distribution function is a parabola with the value of $\Phi(10) = 1$, at $x = 10$ kp, thus above 10 kp all the jars belonging into the set will break. The cumulative distribution function is shown in Fig. 6.

In the first case the breaking process stops and from the values $x_0 = 2$, $x_1 = 2.66$, $x_2 = 4$ and $x_3 = 8$ kp

$$\Phi(x_0) = 0.04, \quad \Phi(x_1) = 0.0711, \quad \Phi(x_2) = 0.16 \quad \text{and} \quad \Phi(x_3) = 0.64$$

is obtained. Substituting these values and

$$m_0 = 1,\ m_1 = 1,\ m_2 = 1,\ m_r = m_3 = 0,\ N = 4$$
and $M = 3$ into Eq. (5)

we obtain

$$P = \frac{4!}{1!1!1!1!} \cdot 0.04^1 \cdot (0.0711 - 0.04)^1 \cdot (0.16 - 0.0711)^1 \cdot (0.64 - 0.16)^0 \cdot$$
$$\cdot (1 - 0.64)^1 = 0.952 \cdot 10^{-3}$$

that is, we can expect that the above process will take place roughly once out of 1000 experiments. In the second breaking process all jars break, the loads are the same as before and now in the last step $m_r = m_3 = 1$ jar will break.

By substitution into Eq. (5) we obtain the result

$$P = \frac{4!}{1!1!1!1!} \cdot 0.04^1 \cdot (0.0711 - 0.04)^1 \cdot (0.16 - 0.0711)^1 \cdot (0.64 - 0.16)^1 \cdot$$
$$\cdot (1 - 0.64)^0 = 1.27 \cdot 10^{-3}$$

It can be seen that the probability of the occurrence of the last event is greater than that of the previous one.

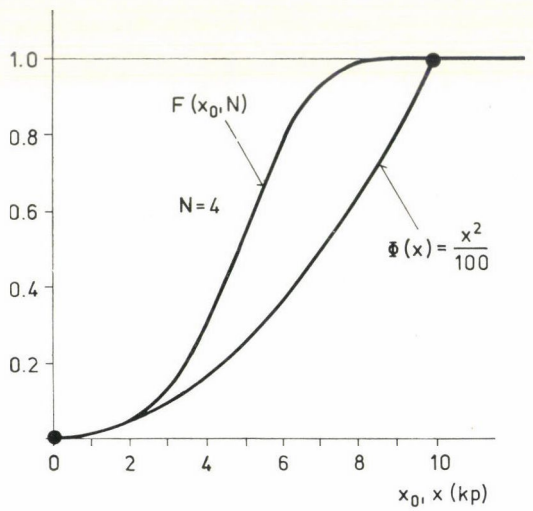

Fig. 6. The cumulative distribution function $\Phi(x) = x^2/100$ and the pertaining $F(x_0, N)$ distribution function for layers consisting of $N = 4$ jars

2.3. *Systematic generation of the possible cases of step-wise breaking*

A method is suggested here for the generation of step-wise breaking which can be used for the computer calculations as described in para. 3. We start with the case when under the primary load, that is in step zero, none of

the jars breaks and here upon all cases are considered in which the jars break only under the primary load up to the stage at which all jars are broken in the order of increasing number of breakages. In this way those cases have been taken into consideration in which the breaking process stops at the first step. Next, the breaking of 1 jar is considered in the first step and the number of jars broken by the primary load are considered in increasing order till all the jars have been considered broken. Then the number of jars broken in the first step is again raised by 1 and the procedure continued as before. When all the possibilities for the primary load (step zero) and for the 1st step have been exhausted, we change to the next step where we take first the case of one jar being broken, and so on.

As an example, we have considered in Table 1 all possible step-wise breaking processes in a layer consisting of $N = 4$ jars and have presented them in the order according to the described procedure. It can be seen that the last event is the breaking of 1 jar in every single step. The table shows the total number of broken jars from the various breaking processes (M) and the proba-

Table 1

Possible cases (events) of the step-wise breaking process for $N = 4$ and the probability of the occurrence of each case (P) at a primary load of $x_0 = 1$ and a cumulative distribution function $\Phi(x) = x^2/100$

Step	Number of breakages per step				Sum of broken jars M	Probability of the occurrence of the processes P
	3	2	1	0		
	Ø	Ø	Ø	0	0	$9.60596 \cdot 10^{-1}$
	Ø	Ø	Ø	1	1	$3.79044 \cdot 10^{-2}$
	Ø	Ø	Ø	2	2	$5.52960 \cdot 10^{-4}$
	Ø	Ø	Ø	3	3	$3.36000 \cdot 10^{-6}$
	Ø	Ø	Ø	4	4	$1.00000 \cdot 10^{-8}$
	Ø	Ø	1	1	2	$8.60160 \cdot 10^{-4}$
	Ø	Ø	1	2	3	$3.02400 \cdot 10^{-5}$
	Ø	Ø	1	3	4	$6.00000 \cdot 10^{-7}$
	Ø	Ø	2	1	3	$6.09778 \cdot 10^{-6}$
	Ø	Ø	2	2	4	$5.40000 \cdot 10^{-7}$
	Ø	Ø	3	1	4	$1.88203 \cdot 10^{-8}$
	Ø	1	1	1	3	$3.48444 \cdot 10^{-5}$
	Ø	1	1	2	4	$4.32000 \cdot 10^{-6}$
	Ø	1	2	1	4	$1.03243 \cdot 10^{-6}$
	Ø	2	1	1	4	$4.60905 \cdot 10^{-7}$
	1	1	1	1	4	$4.97777 \cdot 10^{-6}$

Total number of possible cases $2^N = 2^4 = 16$ $\Sigma P = 1.0000$

bility values pertaining to these processes (P), which were calculated from Eq. (5), by assuming a basic load of $x_0 = 1$ kp and taking into account the cumulative distribution function $\Phi(x) = x^2/100$ of para. 2.2. The totality of events listed in the table make up a complete system of events, since all possible cases from the event of "not a single breakage" to the event of "all jars broken" have been considered. The probability of the occurrence of the complete system is 1, as shown also by the sum ΣP in the table.

It appears from Table 1 that in fact the numbers of the numerical system with base $N+1$ are formed here in increasing order, according to the example the numbers of system with base 5, but certain elements (numbers) of the system are omitted, namely those where the sum of the individual numbers in the places is greater than N (greater than 4 according to the example), since it makes no sense to speak about the breaking of more than N jars.

In addition, a place in the system cannot include zero if the next higher place contains a value other than zero. This is so, because if no jar breaks in a step, none can break in the following step either.

Let us state, omitting verification here, that the number of events (cases) in the complete system is 2^N, in our example $2^4 = 16$. Thus, the number of possible cases increases exponentially with the initial number of jars in the layer, *e. g.* for $N = 10$ the number of possible events is $2^{10} = 1024$. Hence, only computerized calculations can be expected to be useful for the solution of practical problems.

2.4. Determination of the probability density function pertaining to the distribution which describes the step-wise breaking process and of the cumulative distribution function which indicates the average breaking ratio

The density function of the distribution discussed in the previous paragraphs is obtained by adding up the probabilities pertaining to the identical values of total breakages in the layer (M). In this way the density function of a discrete distribution is obtained which can be plotted as shown in Fig. 7. The total number of jars broken in the process is plotted on the horizontal axis and the pertaining (added up) probabilities (P_M) on the vertical axis. Figure 7 shows the values calculated from the data of Table 1. Since the differences between the P_M values are very great these have been plotted on the logarithmic scale.

The cumulative distribution function pertaining to the density function is not shown, the value of the latter are obtained by adding up the values of the ordinates.

The expected value of the distribution is obtained by multiplying the single P_M values by the number of broken jars M and forming the sum of the products.

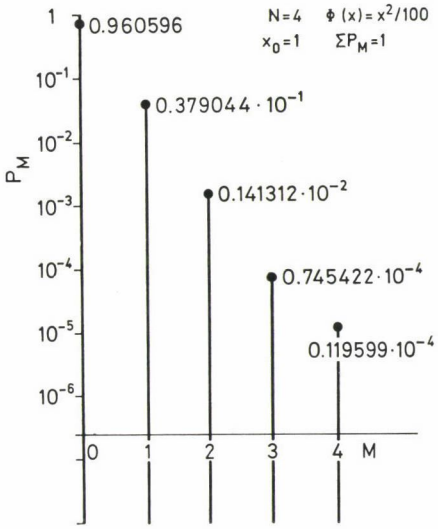

Fig. 7. The probability density function of the distribution describing the step-wise breaking process

The average breaking ratio for a given primary load is obtained by dividing the expected value by N. The average breaking ratio is the function of the primary load and of the initial number of jars, and is therefore designated by the symbol $F(x_0, N)$. It can be calculated from the following equation:

$$F(x_0, N) = \frac{1}{N} \sum_{M=0}^{N} MP_M. \tag{8}$$

For the sake of an example we have presented Fig. 6. In addition to the distribution function $\Phi(x) = x^2/100$ pertaining to the load x, the distribution function $F(x_0, N)$ for step-wise breaking is also shown in this Figure. In agreement with our earlier example we have determined the function $F(x_0, N)$ for a layer consisting of $N = 4$ jars. At low primary loads the average breaking ratio (percentage) with respect to the step-wise breaking process is hardly higher than the value obtained in single breaking experiments with loads equal to the primary load.

At higher primary loads the difference is already considerable. For the determination of the values of $F(x_0, N)$ the computerized method as described in para. 3 has been used.

2.5. Determination of the probability of the simultaneous breaking of several jars

It has already been pointed out in para. 1 that the simultaneous breaking of several jars might be disadvantageous from safety aspects. We shall

next describe a method for the determination of the probability of the breaking of a number of jars larger than permissible from the aspect of safety (M_B) out of N jars in case of a given x_0 primary load, and known number of jars (N) in the layers and provided the cumulative distribution function $\Phi(x)$ is known. In other words, all step-wise breaking processes in course of which more than M_B jars are liable to break are considered unfavourable from the aspect of safety. No doubt the P probability values pertaining to those cases of the complete system of events which have been qualified as unfavourable should be added up. From the point of view of the calculation it is, however, more simple to determine the probability of not more than the specified number of M_B jars being broken and subtracting this from the probability of the occurrence of the complete system of events which is equal to 1. That is, the formula

$$P(M > M_B) = 1 - P(M \leq M_B) = 1 - \sum_{M=0}^{M_B} P_M \tag{9}$$

should be used. The number of events at which not more than M_B jars break is 2^{M_B}

The following example shall serve to illustrate the aforesaid:

The first example is in fact a continuation of the examples related to Table 1 and Fig. 6.

Let us now examine the probability of the breaking processes exceeding the value of $M_B = 3$ at primary loads $x_0 = 1; 1.5; 3; 4.5; 6$ and 7.5 kp. The calculations have been performed on the basis of formulas (5), (6) and (9) by means of the computerized method as described in para. 3. The results are shown in Table 2.

Table 2

The probability of more than $M_B = 3$ jars being broken in course of the step-wise breaking process acting at a primary load x_0 upon a layer consisting of $N = 4$ jars at a distribution function $\Phi(x) = x^2/100$

Primary load x_0	$P(M > M_B)$ $M_B = 3$ $N = 4$
1	$0.1196 \cdot 10^{-4}$
1.5	$0.3065 \cdot 10^{-3}$
3	$0.5000 \cdot 10^{-1}$
4.5	0.3605
6	0.7650
7.5	0.9634

3. Computerized calculation methods for the step-wise breaking process

Next we shall describe the programme written for the electronic desk calculator type HP9820A of HEWLETT–PACKARD Ltd.

The principle of the programme appears from Fig. 8. According to para. 2.3 and in increasing sequence, in agreement with Table 1, the programme produces the numbers corresponding to the various cases of the step-wise breaking process (number-generating programme section). This is being performed up to the permitted number of breakings M_B or all elements of the complete system of events are produced. The programme calculates for a given primary load the probabilities of the single specified events of breaking by using Eq. (5) and determines for a specified M_B value the sum

$$P(M \leq M_B) = \sum_{M=0}^{M_B} P_M$$

in Eq. (9), that is the probability that not more than M_B jars will break. It also calculates for a given primary load the average breaking ratio in the layer from Eq. (8), assuming the formation of large numbers of layers.

The block diagram of the programme is shown in Figs. 9, 10 and 11. The alpha numerical symbols have been adopted from the Hewlett–Packard 9820A Calculator Operating and Programming Manual.

The relationship between the mathematical symbols used so far and the restricted character system of the calculator is as follows:

Mathematical symbol	HP9820A character
N	N
M_B	M
x_0	$X0$
$P(M \leq M_B)$	SP
$F(x_0, N)$	(P)

The construction of the number-generating part of the programme is elaborated in such a way that it generates first in increasing sequence the numbers of the 1st place in the M_B+1 system. Next it produces the numbers of the 1st and 2nd place, but now the base of the numerical system is lowered by one. Thus the highest generated number in places one and two will be M_B-1. The number of the places increases then again by one, but the base number of the system also diminishes by one. In this way the last generated number will contain M_B places and in all places the highest number of the binary system, that is one. The programme generates zero only for the place one, but omits 0 at other places.

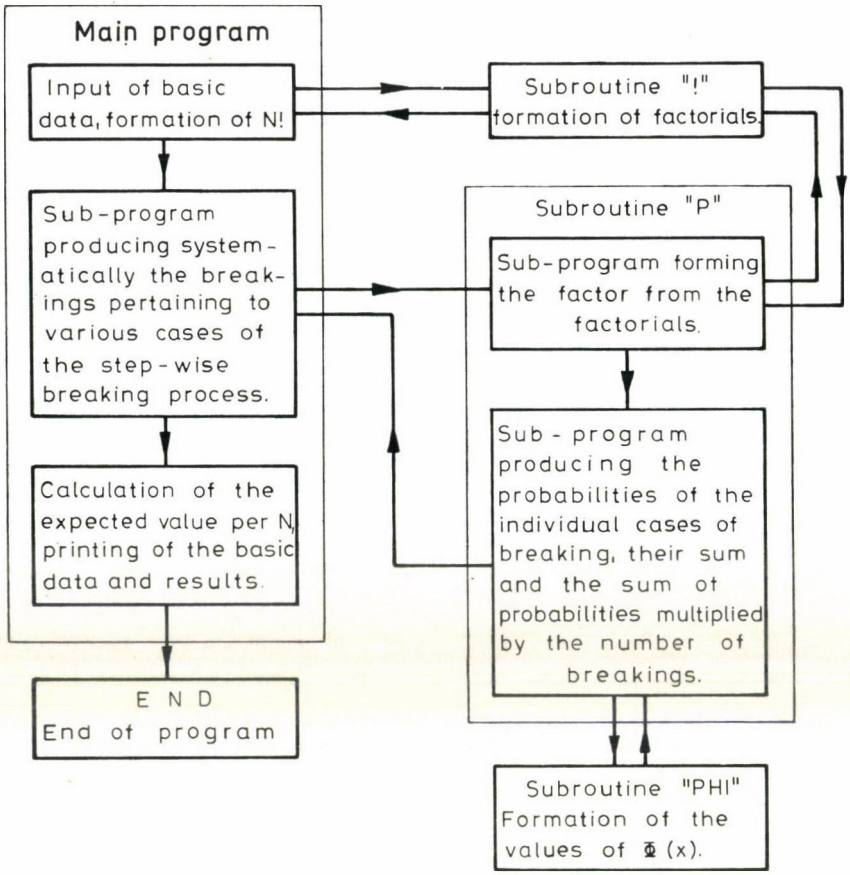

Fig. 8. Block diagram of the computer programme for step-wise breaking processes

In this way a total of

$$K = \sum_{l=0}^{M_B} (M_B + 1 - l)^l \tag{10}$$

numbers are generated by the programme, but of these only those are used in further calculations where the sum of the individual numbers in the places is equal to or lower than M_B. There are, as already mentioned in para. 2.5 a total of $H = 2^{M_B}$ such useful numbers.

For instance, in the case of $M_B = 6$ the total of the generated numbers is:

$$K = 7^0 + 6^1 + 5^2 + 4^3 + 3^4 + 2^5 + 1^6 = 210$$

while there are $H = 2^6 = 64$ useful numbers. In case of $M_B = 10$, $K = 42.312$ and $H = 1024$.

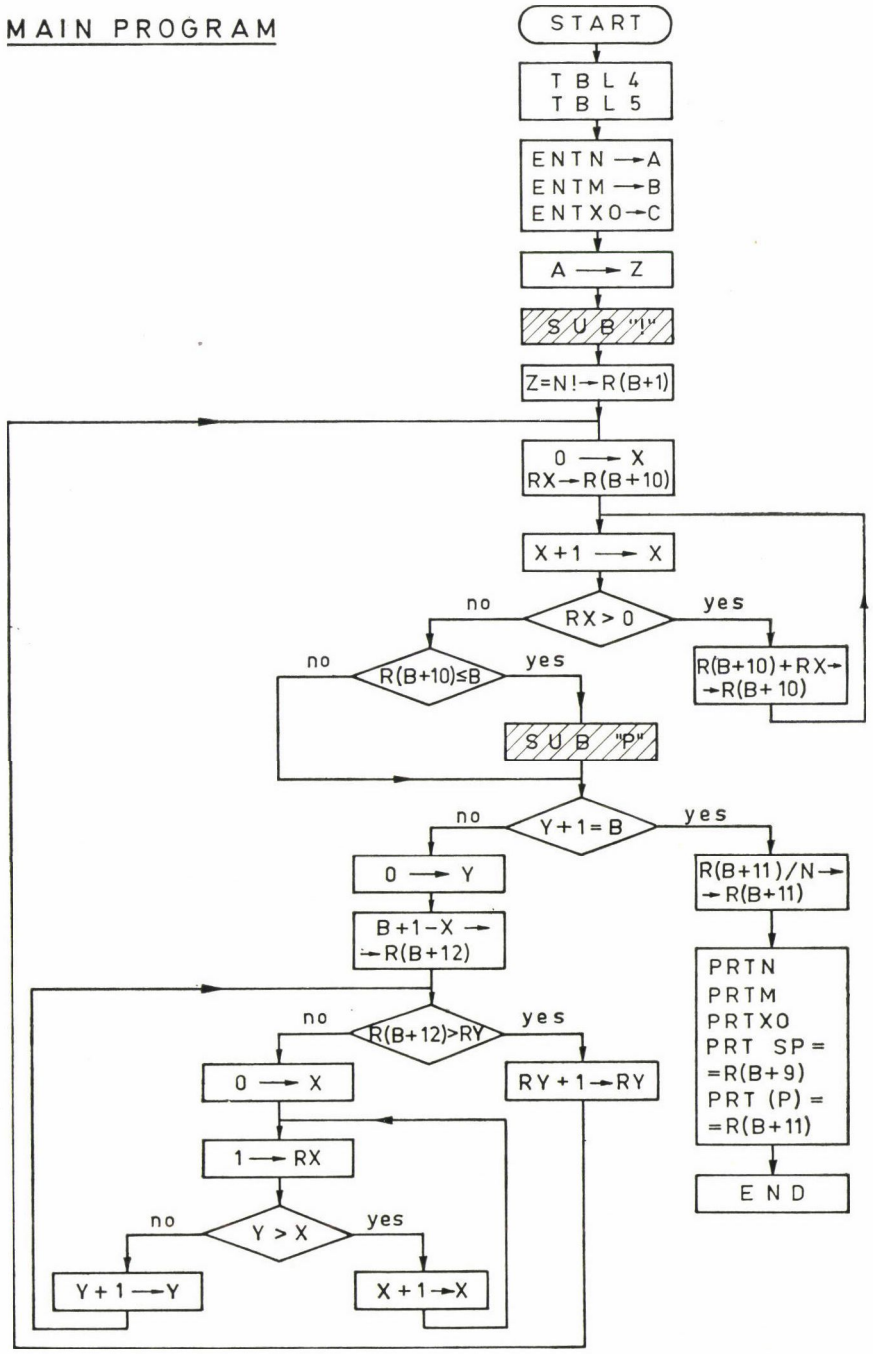

Fig. 9. Block diagram of the "main programme" inside the computer programme for step-wise breaking processes

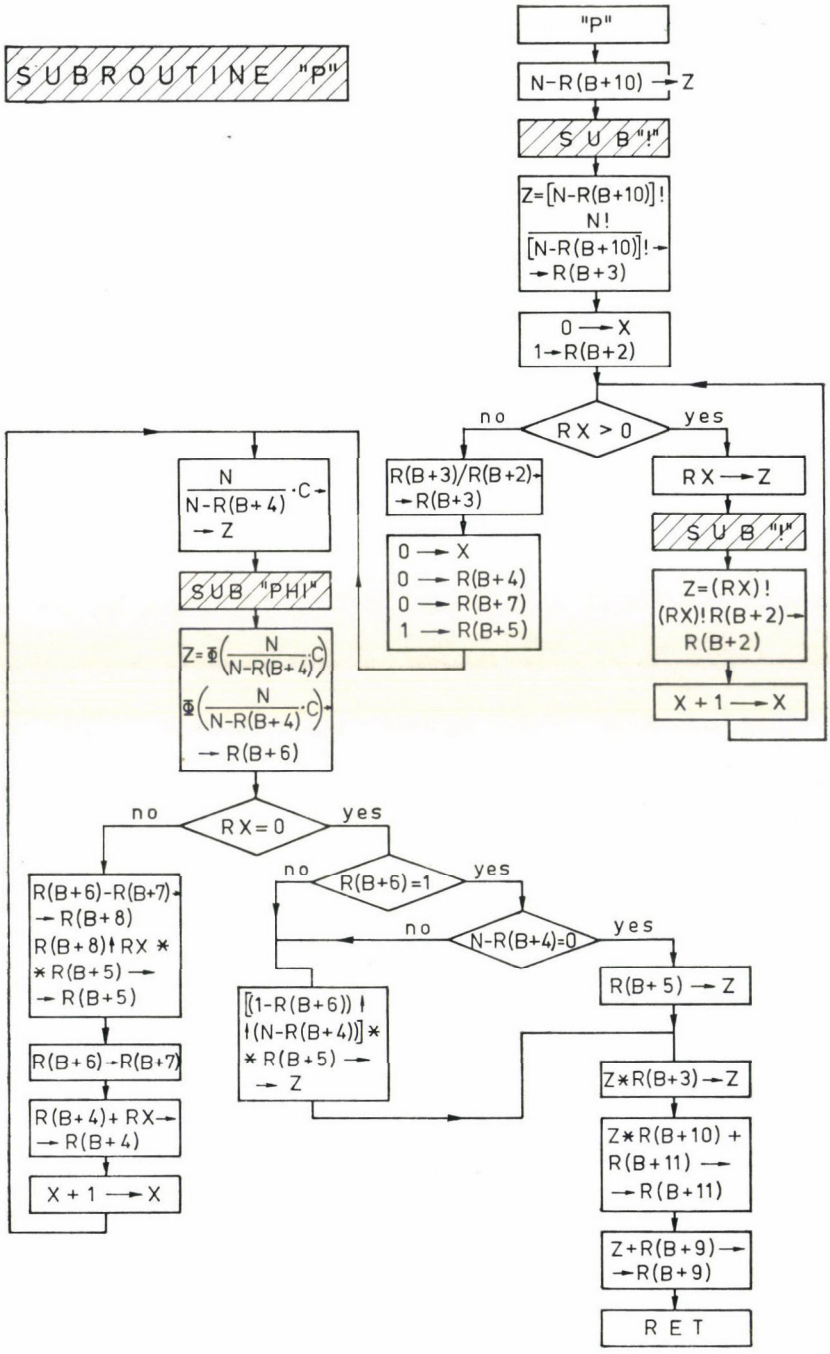

Fig. 10. Block diagram of "P subroutine" in the computer programme for step-wise breaking processes

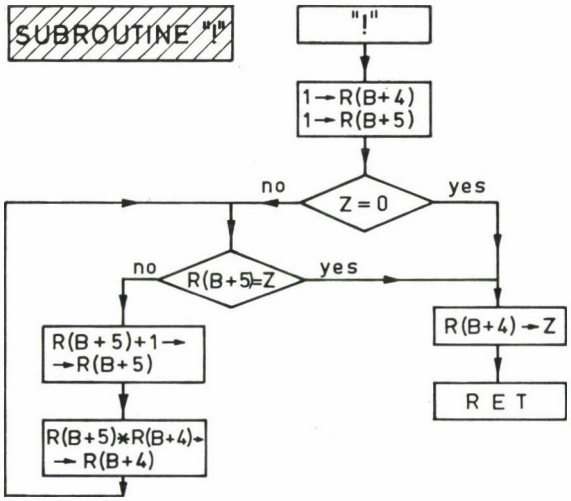

Fig. 11. Block diagram of the "factorial subroutine" inside the computer programme for step-wise breaking processes

4. Investigation of the step-wise breaking process in case of layers consisting of high numbers of jars approaching the infinite

Next, we shall investigate the case when the number of jars in the layer is increased beyond all limits, meanwhile the primary load (x_0) is kept constant.

This procedure is justified for more than one reason. If the number of jars in the layer is high the computerized calculation also becomes very lengthy. Desk calculators are in general not fitted for the calculation of layers with a high number of elements. The calculator, type HP9820A, as mentioned in para. 3 is for instance unsuited for the calculation of layers containing more than 69 jars, since the highest number which can be interpreted in the machine is $10^{99} < 70\,! \approx 10^{100}$. When greater numbers of jars (M_B or N) have to be considered, the number of generated numbers is very high. For instance, for the determination of a single value of the function $F(x_0, N)$ in the case of $N = 33$, $K = 2.9 \cdot 10^{24}$ numbers must be generated. This value was obtained by substitution of $M_B = N = 33$ into Eq. (10). In this case it is impossible to run the programme because of the length of calculation time it would require.

In the canning industry, however, pallet stacks with layers containing more than $N = 100$ jars (5/4 or smaller jars) are used in a large number. From what has been said so far it is fairly obvious that in case of layers with very high numbers of jars an approximate solution should be sought by the calculation method generalized for layers consisting of an infinite number of elements.

4.1. Generalization of the step-wise breaking process for layers consisting of a number of elements approaching the infinite. – Critical primary and average loads

If the number of randomly chosen jars in the layer is raised beyond all limits in such a way that the average load per jar shall remain constant, the cumulative distribution function of the sample will more and more approach the distribution function $\Phi(x)$ of the set. In this sense instead of the step-wise distribution function shown *e. g.* in Fig. 5 the continuous distribution function $\Phi(x)$ as shown in Fig. 12 will be typical for the layer.

Let us now examine the step-wise breaking process for this case. The primary load x_0 will break the jars with a breaking strength lower than x_0 and with a breaking ratio $\Phi(x_0)$. On the remaining jars an average load of

$$x_1 = \frac{x_0}{1 - \Phi(x_0)}$$

will act which will cause the breaking of jars with breaking strengths between x_1 and x_0, their breaking ratio is $\Phi(x_1) - \Phi(x_0)$. The remaining jars will now be subject to the average load of

$$x_2 = \frac{x_0}{1 - \Phi(x_1)}$$

and so on. At each increased value of the average load more jars will break, since among the infinite number of jars in the layer there will be always some liable to breaking. It can be seen that two possibilities exist: either all jars break gradually, or the process approaches through an infinite number of steps a limit value, thus in this sense the process terminates. These two possibilities are illustrated in Figs. 12 and 13.

If the breaking process terminates, the series of the values of both x_0, x_1, x_2, x_3, . . . and $\Phi(x_0)$, $\Phi(x_1)$, $\Phi(x_2)$, $\Phi(x_3)$ . . . tend towards a limit value. Let us give the symbol x_e to the limit value of the first series and $\Phi(x_e)$ to that of the second series, then we have the relationship between the two expressed as

$$x_e = \frac{x_0}{1 - \Phi(x_e)} \tag{11}$$

The validity of Eq. (11) can be demonstrated as follows: in step j the value $\Phi(x_j)$ will give the ratio of all previously broken jars, and the average load here will be

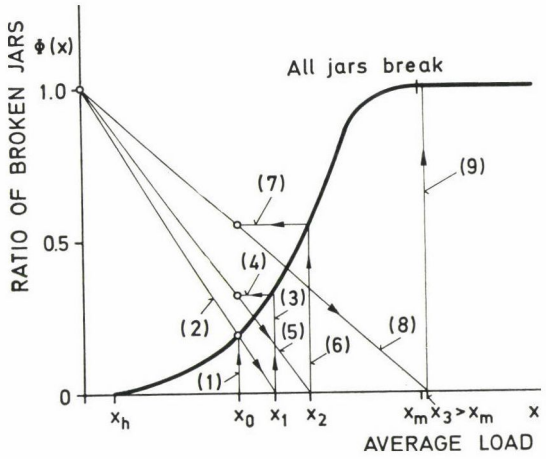

Fig. 12. Illustration of the step-wise breaking process in case of an infinite number of jars in the layer and when all jars break

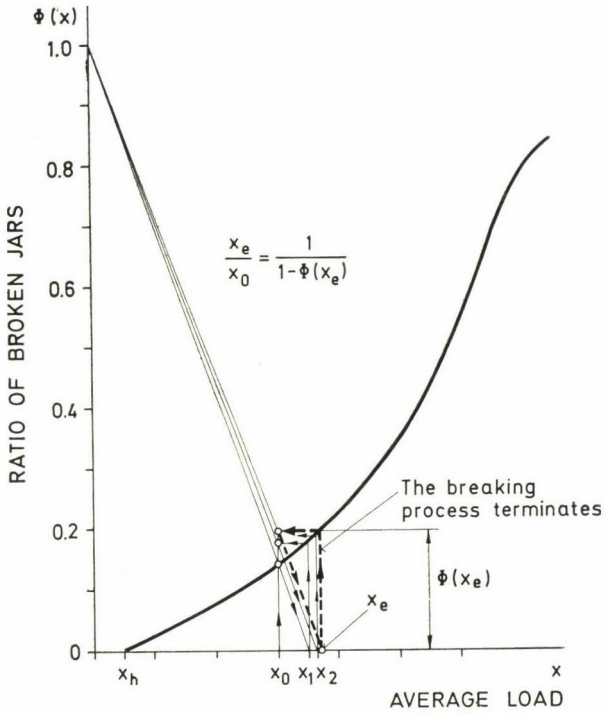

Fig. 13. Illustration of the step-wise breaking process in case of an infinite number of jars in the layer and when the breaking process terminates

$$x_{j+1} = \frac{x_0}{1-\Phi(x_j)} \tag{12}$$

Here the value of x_{j+1} can be expressed in the form

$$x_{j+1} = x_j + \Delta x_j$$

and with this expression Eq. (12) can be written in the form

$$x_j + \Delta x_j = \frac{x_0}{1 - \Phi(x_j).} \tag{13}$$

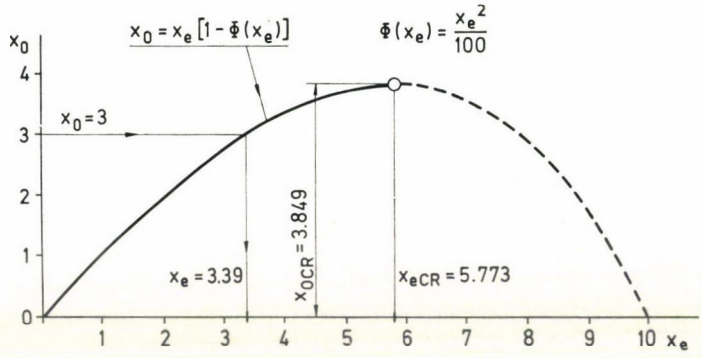

Fig. 14. Primary load (x_0) *vs.* the limit value of the average load (x_e) for a distribution function $\Phi(x) = x^2/100$

When the breaking process terminates, the increase of j beyond all limits gives on the left side of Eq. (13) the limit values

$$\lim_{j\to\infty} \Delta x_j = 0, \quad \lim_{j\to\infty} x_j = x_e$$

That is, the difference between two successive average loads tends towards zero. On the right hand side of Eq. (13) $\Phi(x_j)$ tends obviously towards the limit value $\Phi(x_e)$.

From Eq. (11) it is possible to determine x_e and $\Phi(x_e)$ by writing this equation in the form

$$x_0 = x_e[1-\Phi(x_e)] \tag{14}$$

and plotting x_0 as the function of x_e. Here the abscissa x_e pertaining to the ordinate x_0 will represent the average load at the limit of the process and $\Phi(x_e)$ the pertaining breaking ratio. The method is illustrated in Fig. 14 which has been plotted by applying the distribution function of the examples in paras. 2.2, 2.3, 2.4 and 2.5.

For instance, it can be seen from the figure that an average load of $x_e = 3.39$ belongs to the primary load $x_0 = 3$ kp which involves a breaking ratio $\Phi(x_e) = 3.39^2/100 = 0.115$ and means that the breaking process will terminate at these values.

The maximum of curve x_0 has been given the symbol x_{0CR} and the pertaining x_e the symbol x_{eCR}. These values play a very important role as will be shown below.

In the case of $x_0 > x_{0CR}$ a real solution of Eq. (14) can be interpreted only as $x_e \to \infty$, and $\Phi(x_e) \to 1$ thus if $x_0 > x_{0CR}$ all jars in the layer will break.

Consequently, x_{0CR} is that critical (maximum) primary load which if exceeded the breaking process will no longer stop. For the case illustrated in Fig. 14 the values

$$x_{0CR} = 3.8487 \text{ and } x_{eCR} = 5.773$$

were determined by the solution of the equation $\frac{dx_0}{dx_e} = 0$, through the following steps:

$$x_0 = x_e[1-\Phi(x_e)] = x_e[1-x_e^2/100], \frac{dx_0}{dx_e} = 1-3x_e^2/100 = 0$$

and from this

$$x_{eCR} = (100/3)^{1/2} = 5.773$$

and

$$x_{0CR} = 5.773\ (1-5.773^2/100) = 3.8487.$$

The downward sloping section of curve x_0 after the maximum is no longer needed and is therefore shown by a dotted line.

The curve x_0 might have more than one apex, in such cases the highest of the apices will give the critical primary and critical average loads. Such a case is illustrated in Fig. 15 which represents a mixture of two types of glasses with different breaking strengths (see also Fig. 4). At the $x_{0A} = 4$ kp primary load corresponding to the first apex the breaking ratio is $\Phi(x_{eA}) = 0.333$ and the average load rises to $x_{eA}=6$ kp.

If the primary load is increased by an infinitesimal value, the breaking process will stop only at an average load of $x_{eB} = 10$ kp and $\Phi(x_{eB}) = 0.6$.

The critical primary load is $x_{0CR} = 4.9$ kp to which $x_{eCR} = 12.5$ kp is assigned. In the figure the section of the curve x_0 between x_{eA} and x_{eB} has been drawn with a dashed line, since the step-wise breaking process will by all means overpass this section; it cannot terminate in it (Fig. 15).

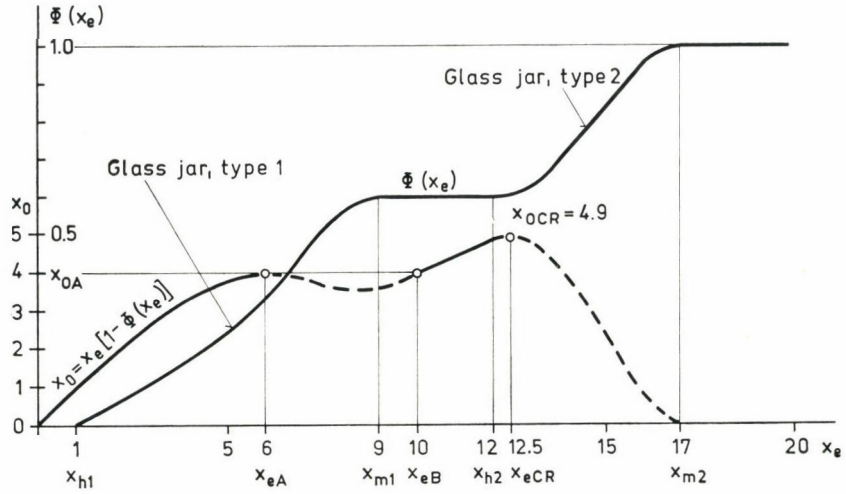

Fig. 15. Cumulative distribution function of a set consisting of a mixture of two types of glass jars and the primary load (x_0) *vs.* the limit value of the average load (x_e). There is no overlapping between the ranges limited by the minimum and maximum breaking strengths

4.2. Determination of the distribution function $F(x_0, N \to \infty)$ pertaining to the step-wise breaking process of a layer containing an infinite number of elements

In para. 2.4 we have described the method for the determination of the distribution function $F(x_0, N)$ for the average breaking ratio in layers consisting of N jars by accounting for the step-wise breaking process.

In case of layers containing an infinite number of elements (jars) the situation is much simpler, since to the primary load x_0 an average breaking ratio

$$F(x_0, N \to \infty) = \Phi(x_e) \tag{15}$$

can be assigned, for in all layers containing an infinite number of elements the average load will be x_e and the breaking ratio $\Phi(x_e)$.

In the following discussion we shall use instead of the symbol $F(x_0, N \to \infty)$ the simpler expression $F(x_0, \infty)$. The function $F(x_0, \infty)$ can be determined as follows: various x_e values are taken beginning from $x_e = x_h$ in increasing sequence and the pertaining $\Phi(x_e)$ values and next the x_0 values from Eq. (14) are determined. The values obtained as x_0 are plotted on a graph paper on the abscissa and the pertaining $\Phi(x_e)$ values on the ordinate. The values of $\Phi(x_e)$ are given the symbol $F(x_0, \infty)$ and the procedure is continued till the values x_{OCR} and x_{eCR} are reached. The example linked to Fig. 14 is continued on Fig. 16. This figure shows also the $F(x_0, N)$ functions pertaining to layers

having $N = 1$, 2 and 4. We wish to note that in the case of a layer consisting of $N = 1$ jar

$$F(x_0, N=1) = \Phi x_0.$$

since we are dealing here with an individual breaking experiment.

Fig. 16 shows clearly the character of the curve $F(x_0, \infty)$. At the beginning the curve does not deviate markedly from the other $F(x_0, N)$ curves, but when the critical primary load (x_{0CR}) is reached the entire layer breaks suddenly. Strictly speaking at the critical primary load the breaking ratio is

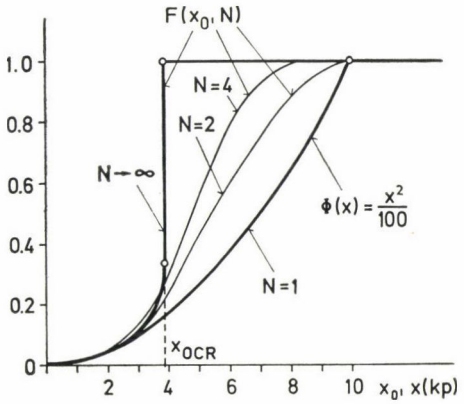

Fig. 16. The cumulative distribution function $\Phi(x) = x^2/100$ and the pertaining distribution functions $F(x_0, N)$ in case of $N = 2$, 4 and $N \to \infty$.

$$F\,(x_{0CR}, \infty) = \Phi\,(x_{eCR})$$

which in the example illustrated in Fig. 16 is

$$F\,(x_{0CR}, \infty) = 0.333.$$

However, when the critical primary load is increased by an infinitesimally small value, the entire layer will break, since the curve representing the function $F(x_0, \infty)$ has a vertical tangent at the critical primary load. This last statement can be easily proved, since the differential quotient of the inverse function of $F(x_0, \infty)$ must be zero at the values $F(x_{0CR}, \infty)$ and x_{0CR}. In fact: since $\mathrm{d}F(x_0, \infty) = \mathrm{d}\Phi(x_e)$ according to Eq. (15) and the well-known rule of forming the differential quotient of a function's function has been applied

$$\frac{\mathrm{d}x_0}{\mathrm{d}F(x_0, \infty)} = \frac{\mathrm{d}x_0}{\mathrm{d}\Phi(x_e)} = \frac{\dfrac{\mathrm{d}x_0}{\mathrm{d}x_e}}{\dfrac{\mathrm{d}\Phi(x_e)}{\mathrm{d}x_e}} \,. \tag{16}$$

The right hand side of Eq. (16) is equal to zero if $x_e = x_{eCR}$ and $x_0 = x_{0CR}$, since dx_e in the numerator is zero at the critical values, as has been shown in para. 4.1, while the value of $d\Phi(x_e)/dx_e$ in the denominator is never zero at the critical values. This last statement is true even when the function $\Phi(x_e)$ happens to have a horizontal section, for x_{eCR} cannot lie between the limits of this section. This can be proved by converting the expression $d\Phi(x_e)/dx_e$ with the help of Eq. 14, next substituting the values x_{0CR} and x_{eCR}. In this way we obtain

$$\left[\frac{d\Phi(x_e)}{dx_e}\right]_{x_e=x_{eCR}} = \frac{1}{x_{eCR}}\left[1 - \Phi(x_{eCR})\right] \tag{17}$$

The right side of this expression is equal to zero only when $\Phi(x_{eCR}) = 1$, but in this case the breaking process does not stop.

In case of layers containing a large number of jars, as is common in the canning industry, the critical primary load has thus a predominant role: the primary load must not approach the critical value. For layers containing large numbers of jars it is sufficient to perform the experiments concerning the breaking of individual jars up to the load x_{eCR}, whereby the range of the applied forces can be considerably narrower.

It is an important fact worth considering that in case of layers containing infinite number of elements there are only two answers to the question: what is the probability that at a given primary load the breaking process will stop at or below a given breaking ratio namely it will either certainly stop (probability 1), or the jars are certain to break at a higher ratio (probability of the process being terminated is zero).

If, however, the breaking ratio permissible from the aspect of safety is specified, we have also specified, in agreement with the aforesaid, the primary load which must not be exceeded.

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NEW EXPERIMENTAL AND MATHEMATICAL METHODS CONCERNING STRENGTH PROBLEMS IN PALLET STACKS OF CANNING JARS

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PART II. — INDUSTRIAL EXAMPLES AND EXPERIMENTAL DESIGNS

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In Part I (KÖRMENDY & HAZAY–BALTHAZÁR, 1976) theoretical and mathematical problems were discussed. This part presents examples related to practice in the canning industry, such as determination of the average breaking ratio of columns made up of individual stacks based upon the results of breaking tests, probability of the occurrence of a number of broken jars higher than permissible from safety aspects in the lowest layer of a column of stacks, determination of critical primary and average loads (x_{0CR} and x_{eCR}) for various types of canning jars.

Next, the aspects to be considered in relation to breaking strength are analyzed in greater detail than in Part I and an experimental basic principle is given for the determination of the unfavourable arrangements in the stacks from the aspect of safety. Experimental designs are suggested for tests in connection with the individual breaking of glass jars. The axial compression forces suggested for breaking tests for vacuum-free jars, vary between 35 and 450 kp for 5/4 Comecon jars. For 5/4 NHC jars the same forces vary between 35 and 400 kp, and for 5/1 N jars between 110 and 800 kp.

This paper is the continuation of Part I of the same title (KÖRMENDY & HAZAY-BALTHAZÁR, 1976). The following examples are intended to show the application of the theoretical considerations in Part I and cannot be used for the justification of industrial procedures. The list of literature contains some publications which have already been mentioned in Part I in order to save the reader the trouble to have to refer back to Part I. The symbols used are listed at the end of this Part.

All that is being said in Parts I and II about the step-wise breaking process in layers containing finite or infinite number of jars are the authors' original ideas. The same is true for the method suggested for the determination of the average breaking ratio and for the essential parts of the programme written for the calculator type HP9820A, for the experimental methods used for the determination of cases unfavourable from the aspect of safety and for the suggestions concerning the way in which the vacuum should be taken into consideration in the breaking tests.

The method described in para. 2.2 of Part II for the determination of the confidence intervals has in essence been known before, but treated in a concise way by the pertaining literature, which induced the authors to present it in somewhat greater detail.

1. Examples

1.1. Determination of the average breaking ratio in stacks heaped on top of each other

For the determination of the average breaking ratio in stacks heaped on top of each other first of all the distribution function $F(x_0, N)$ — which accounts for the step-wise breaking process — must be known. By means of this function the ratio of broken jars in relation to the total set $[U]$ is obtained by taking into consideration the step-wise breaking process at a loading force (primary load) x_0 per jar. The method for calculating the relation between the distribution function $\Phi(x)$ obtained from breaking experiments on individual jars and the distribution function $F(x_0, N)$ has been described in para. 2.4 of Part I.

The procedure of the determination of the average breaking ratio (percentage, Z) involves the determination of the actual value of the primary load (x_{0K}) in every layer of the stacks heaped on top of one another, then the calculation of the value of the pertaining average breaking ratio $F(x_{0K}, N)$ and the formation of their average from the formula

$$Z = \frac{100}{R} \sum_{K=1}^{R} F(x_{0K}, N) \tag{1}$$

where R stands for the number of layers heaped one on top of the other. Let us illustrate this by the following example:

According to the experimental results of FERENCZY and KUNOS (1973) in the INSTITUTE FOR MATERIAL TRANSPORT AND PACKAGING TECHNIQUE, Budapest, cumulative distribution function $F(x_0, N = 33)$ of 5/1 N type jars filled with canned goods can be approximately described in the $x_0 < 135$ kp range with the help of the expression

$$F(x_0, N = 33) = 5.64\ (x_0 - 100)^{1.28} \cdot 10^{-5}.$$

Let us now examine the value of the average breaking ratio when 4 stacks, each consisting of 4 layers are heaped one on top of the other.

Calculating from the bottom layer upwards, the primary load on the jars and the pertaining $F(x_0, N = 33)$ function will have the following values in each layer:

Number of layer	Primary load (x_{0K}), kp	The value of $F(x_{0K}, N=33)$ pertaining to the primary load
1	108	$8.04 \cdot 10^{-4}$
2	101.35	$0.83 \cdot 10^{-4}$
3 – 16	<100	0
$R = 16$	$\sum_{K=1}^{16} F(x_{0K}, N = 33) = 8.87 \cdot 10^{-4}$	

From the 3rd layer upward the loads were no longer determined, since for these loads $F(x_{0K}, N = 33) = 0$.

From Eq. (1) we obtain for the average breaking ratio:

$$Z = 100\frac{1}{16} \cdot 8.87 \cdot 10^{-4} = 5.54 \cdot 10^{-3} \text{ per cent} = 0.0554‰.$$

In the calculations the weight of one filled jar was taken as 6.5 kp and the number of jars in each layer as 33. The weight of the pallet was 23.3 kp and that of one load distributing plate 3.5 kp.

1.2. Probability of the breaking of a number of jars larger than permissible in the bottom layer of the column of stacks

In the following example we shall investigate the following case:

Let us heap 4 stacks of 5/1 N jars one on top of the other. In each layer of the stack there are 33 jars. Let us now assume that in the range between 0 and 625 kp the cumulative distribution function is in agreement with Fig. 3 in Part I:

$$\Phi(x) = 7.45(x-100)^{1.142} \cdot 10^{-5}. \tag{2}$$

The question now is: what is the probability of more jars being broken in the bottom layer than $M_B = 6$, the number permissible from the aspect of safety.

The primary load in the bottom layer is, from the weight on it, $x_0 = 108$ kp (see example in para. 1.1).

The problem can be solved with the help of the considerations and computer programme described in paras. 2.4, 2.5 and 3 of Part I. The programme was run on the electronic desk calculator type HP9820A (HEWLETT–PACKARD Ltd.). The following result was obtained:

$$P(M > M_B) = 1 - P(M \leq M_B) = 1 - \sum_{M=0}^{M_B} P_M = 5 \cdot 10^{-10}.$$

If we assume that Eq. (2) is a reliable description of the jars filled with canned good and that in one year about 65 000 stacks heaped in the above described manner are formed, then the case when more than 6 jars break in the bottom layer will occur in every $3 \cdot 10^4$th year.

1.3. Determination of critical loads from currently available experimental data

Next, we shall determine the critical loads by making use of the results of FERENCZY and KUNOS (1973).

We shall omit a closer analysis of the experiments and consider the cumulative distribution functions obtained from them as the true distribution function $\Phi(x)$ or $\Phi(x|p = 0)$ of the set. The following types of jars were included in the given number into the tests:

	Number of jars
5/4 Comecon jar, new, filled with canned goods	200
5/4 Comecon jar, new, empty	108
5/4 NHC jar, new, filled with canned goods	200
5/4 NHC jar, used, empty	100
5/1 N jar, 52.6 % new, 47.4 % used, filled	380
5/1 N jar, 50 % new, 50 % used, empty	200

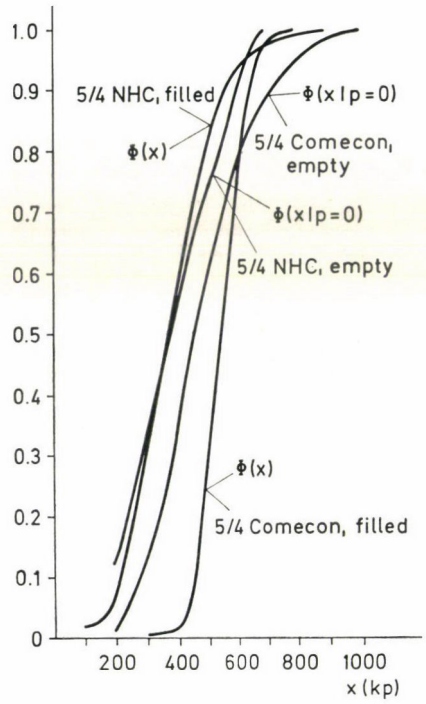

Fig. 1. Cumulative distribution functions for 5/4 Comecon and 5/4 NHC glass jars from the preliminary experiments of FERENCZY and KUNOS (1973)

Figure 1 shows the distribution functions $\Phi(x)$ and $\Phi(x|p = 0)$ for 5/4 jars and Fig. 2 the primary load x_0 as a function of x_e for the same type of glass jars.

Figures 3 and 4 present the same functions for 5/1 N jars, supplemented with the function $F(x_0, \infty)$ for filled 5/1 N jars in Fig. 3 as an example.

In Table 1 the critical values, obtained from Figs. 3 and 4 the expected values of the distribution functions and the minimum breaking strengths (x_h) are given. It appears from this Table that the value of x_{eCR} is always smaller than the expected value.

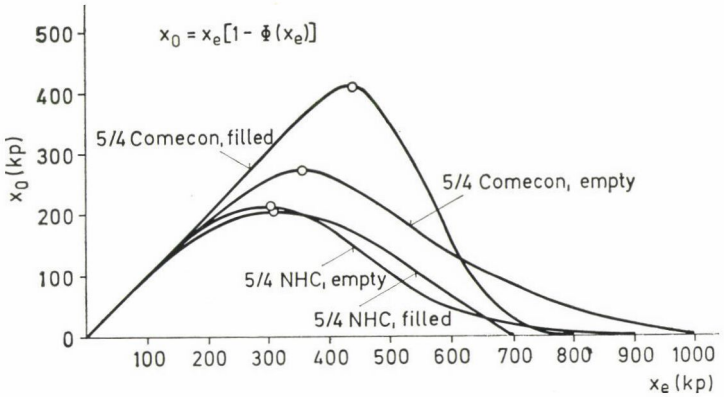

Fig. 2. Diagrams pertaining to the distribution functions in Fig. 1, representing the primary load (x_0) *vs.* the limit value of the average load (x_e)

Table 1

Characteristic data of various types of glass jars
Calculated from the tests of FERENCZY and KUNOS (1973)

Type of glass jar	Minimum breaking strength x_h, kp	Critical primary load x_{0CR}, kp	Critical value of the average load x_{eCR}, kp	Expected value of the distribution function of the sample $\bar{z}$, kp
5/4 Comecon, new, filled	32	410	438	546
5/4 Comecon, new, empty	–	275	355	487
5/4 NHC, new, filled	28	205	310	394
5/4 NHC, used, empty	–	215	305	391
5/1 N, new + used, filled	100	598.5	725	850
5/1 N, new + used, empty	–	470	720	942.5

It appears from Fig. 3 that at smaller primary loads there is hardly any difference between the values of $F(x_0, \infty)$ and $\Phi(x)$, while at the critical primary load suddenly the entire layer breaks (collapses).

Assuming the validity of Eq. (2) in para. 1.2 ($100 < x < 625$ kp), to an average load of $x_e = 400$ kp the primary load

$$x_0 = x_e[1-\Phi(x_e)] = \\ = 400[1-7.45(400-100)^{1.142} \cdot 10^{-5}] = 380 \text{ kp}$$

is assigned. The average breaking ratio in the layer will be

$$F(x_0, \infty) = \Phi(x_e) = 7.45(400-100)^{1.142} \cdot 10^{-5} = 5.04 \cdot 10^{-2}.$$

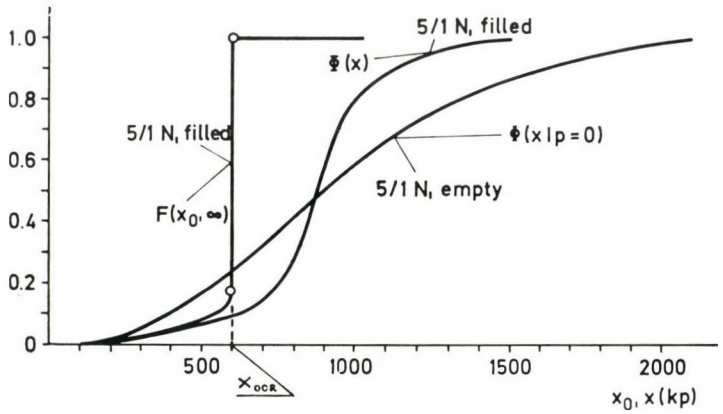

Fig. 3. Cumulative distribution functions for 5/1 N jars from the preliminary experiments of FERENCZY and KUNOS (1973). The average breaking ratio $F(x_0, \infty)$ for jars filled with canned goods is also shown

The value of the cumulative distribution function obtained from individual breaking experiments will be

$$\Phi(x_0) = 7.45(380–100)^{1.142} \cdot 10^{-5} = 4.67 \cdot 10^{-2}$$

at the primary load, and the deviation of $F(x_0, \infty)$ from $\Phi(x_0)$ will be only about 7.5%.

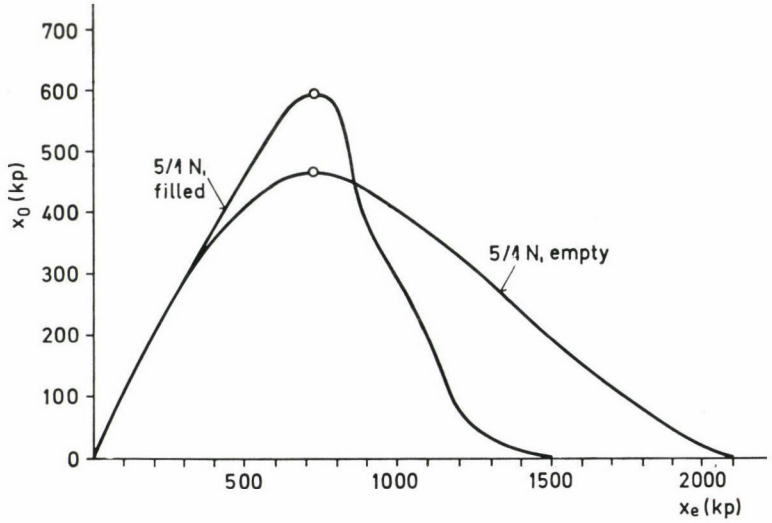

Fig. 4. Diagrams pertaining to the distribution functions in Fig. 3, representing the primary load (x_0) *vs.* the limit value of average load (x_e)

A similar calculation or the measurement of the corresponding ordinates in Figs. 3 and 4 at $x_{eCR} = 725$ kp will lead to the values:

$$x_{0CR} = 598.5 \text{ kp}, \quad F(x_0, \infty) = \Phi(x_e) = 0.175$$

and $\Phi(x_0) = 0.113$. The difference between the last two values is about 40%, while it is enough to exceed the value x_{0CR} by an infinitesimal value to cause the entire layer to break.

2. Experimental design

As already mentioned in para. 1 of Part I the processes related to the breaking strength of glass jars must be considered from two aspects. One aspect is to keep the average breaking ratio at the lowest possible value in case of glass jars filled with canned food, while the other is to avoid any accident or greater damage due to the simultaneous breaking of the jars.

So far the first aspect has been generally neglected in experiments for the canning industry. FERENCZY and KUNOS (1973) when dealing with the individual breaking of jars neglected the range of loading forces which are actually present in the columns of stacks of canned goods. In the experimental plan to be described below we intended to supplement for this omission. Thus, in the six series of experiments performed by FERENCZY and KUNOS (1973) with 5/1 N jars the lowest loading force was 100 kp only in a single case, in the others it was 200, 300, 500 and 600 kp, resp. We have, on the other hand, determined for this type of glass the lowest loading force as 100 kp and instead of using 100, 200 and 300 kp force-intervals, we applied up to 150 kp 10 kp and then 50 kp intervals (see para. 2.2). Registration of the breaking waste in the stores is also very important because of the great value of the goods.

With respect to the second aspect we have already described one of the possible methods of calculation together with some pertaining examples (Part I, para. 2.5, Part II, para. 1.2). This method is, however, only an approximation. In our opinion the exact method would be to produce systematically under experimental conditions the cases (events) which are unfavourable from the aspect of safety and to calculate the probability of their occurrence, *i. e.* to determine the subset of cases which are unfavourable from the aspect of safety and to relate it to the entire system of events.

The suggested experimental method consists in the preparation of a heap of stacks on top of each other, assembled of metal jars imitating glass jars, placing an appropriate number of real glass jars among them in the appropriate arrangement and breaking them in the course of the experiment with a hammer or some other useful tool. In this way it is possible to produce the

various unfavourable cases. The glass jar imitations made of metal are not used for load measurement, but are needed for their strength, or else the spontaneous breaking of the glass jars might interfere with the experimental plan.

2.1. *General structure of the experimental design for the individual breaking of glass jars*

The fundamental principles underlying the experiments concerning the individual breaking of glass jars have already been discussed in para. 1 of Part I, thus are assumed to be known to the reader. The aim of the experiments concerning the individual breaking of glass jars is the determination of the cumulative distribution function $\Phi(x)$ of the set $[U]$ with the best possible approximation. This, however, would require the inclusion of a large number of randomly chosen jars filled with canned goods and the breaking of the same together with their costly contents. The situation is complicated by the fact that the distribution function of the vacuum as of a probability variable might differ from one group of products to the other, when unfortunately the determination of the distribution function $\Phi(x)$ must be carried out separately for the groups of products which differ with respect to the vacuum in their jars.

The following methods are suggested for the elimination of these difficulties:

a) For the individual breaking tests the jars should be filled with water and no vacuum shall be applied. In this way the conditional cumulative distribution function $\Phi(x|p = 0)$ is obtained. For these experiments the apparatus shown in Fig. 2 of Part I can be used.

b) The experiments according to para. *a)* shall be repeated at several expediently chosen $p =$ constant values, whereby an adequate number of conditional cumulative distribution functions $\Phi(x|p =$ constant) are obtained, when instead of the apparatus shown in Fig. 2, Part I the modified arrangement illustrated in Fig. 5 has to be used.

c) The cumulative distribution function $\Psi(p)$ of the specified type of jar should be determined, if necessary according to the group of products. For these tests pressure gauges with appropriate piercing mandrels are available. The rather simple tests have to be performed before the jars have been opened for consumers' use.

d) The cumulative distribution function $\Phi(x)$ can now be determined from the equation

$$\Phi(x) = \int_0^x \left[\int_0^{p_{\max}} \psi(p)\, \varphi(x|p) \mathrm{d}p \right] \mathrm{d}x, \tag{3}$$

practically by means of the approximating method based on Eq. (3).

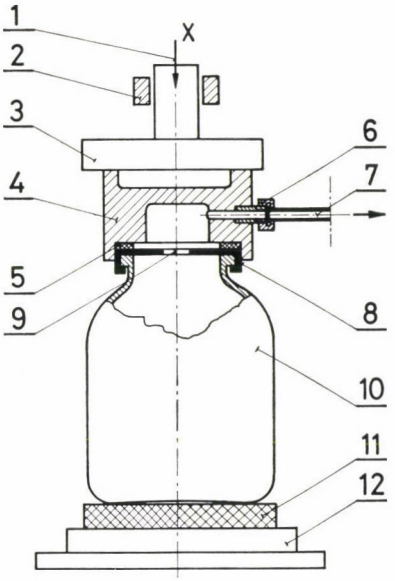

Fig. 5. Device for the breaking tests of glass jars: determination of the effect of vacuum on the breaking strength of glass jars. 1: loading force; 2: shaft locating bearing; 3: pressure plate; 4: load transfer and evacuating head; 5: sealing ring; 6: pipe joint; 7: pipe-line to the tank under vacuum; 8: closure of the jar; 9: hole on the cap; 10: canning jar; 11: cushioned pad; 12: supporting plate

In Eq. (3) $\psi(p)$ is the probability density function of the cumulative distribution function $\Psi(p)$ and $\varphi(x|p)$ that of the conditional distribution function $\Phi(x|p = \text{constant})$. For the confirmation of this correlation we refer to PRÉKOPA's (1962) work.

The above method has the advantage that only empty jars need to be broken and that the distribution function $\Phi(x|p = \text{constant})$ and the density functions $\varphi(x|p)$ have to be determined only once for each type of jar and the latter can be multiplied according to Eq. (3) by different $\psi(p)$ density functions.

The breaking force x_i as a probability variable is not independent of p_i. Introducing the breaking force for vacuum-free jars y_i as a new probability variable, identity exists between its distribution function and the conditional distribution function $\Phi(x|p = 0)$. It is evident that y_i is independent of p_i and x_i can be expressed as the function of the two. It might be advisable to clarify this relationship, since it would probably greatly simplify the calculations.

For the actual performance of the tests the publications on this subject, as listed in the references of Literature of Part I should be taken into consideration.

1.2. Experimental design for the determination of the distribution function $\Phi(x|p = 0)$ characteristic of the breaking strength of vacuum-free jars from the currently available preliminary experimental data

The experimental design has been worked out for the following types of glass jars: (KARDOS & SZENES, 1972):

5/4 Comecon jars, empty
5/4 NHC jars, empty
5/1 N jars, empty

The experimental design was elaborated on the basis of the experiments of FERENCZY and KUNOS (1973). These data are considered as preliminary experiments, since very few jars (108, 100 and 200 jars of the above specified types) were included in the tests.

Not even this small number of glass jars can be considered as randomly chosen, since the 5/4 Comecon jars were new, while the 5/4 NHC jars were all used ones and among the 5/1 N jars there was about 50% of new and 50% of used jars (see para. 1.3). We nevertheless decided to use these data.

The experimental design concerns the individual breaking of jars. The suggested values of the loading forces are given in Table 2. These values were chosen on the basis of the data in Table 1 and it appears that the minimum loading force is close for all types of jars to the x_h value for the same type in Table 1, while the maximum loading force is always greater than the value of x_{eCR}.

It has to be added that all the available x_h values refer to full jars, consequently we were compelled to use these. Forces were increased in smaller intervals in the range of small loading forces for this was the range most interesting from the aspect of the breaking ratio, as has already been pointed out before. In Table 2 informative values are given for the primary load in the bottom layer of the stacks on pallets in case of a column consisting of 4 stacks heaped one on top of the other and jars filled with canned food.

Table 2

Loading forces suggested for the breaking tests of empty glass canning jars

Type of glass jar	Suggested values of the loading force for the breaking test, kp	Informative value of the primary load on filled jars in the bottom layer of a column of 4 stacks, kp	Number of jars in a layer of the stack
5/4 Comecon, empty	35, 40, 45, 50, 100, 150, 200, 250, 300, 350, 400, 450	35	112
5/4 NHC, empty	35, 40, 45, 50, 100, 150, 200, 250, 300, 350, 400	38	112
5/1 N, empty	110, 120, 130, 140, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800	108	35

After the determination of the loading forces the number of jars included into the tests must be found. This might be done as follows:

By means of the experiments performed at a given loading force we wish to determine the cumulative distribution function $\Phi(x|p = 0)$ of a set consisting of an infinite number of elements. Let us give the symbol n to the number of jars used in the experiment and k to the number of jars broken by the loading force x, then the value $\Phi(x|p = 0)$ can be approximated by the frequency k/n. For the sake of simplicity the value $\Phi(x|p = 0)$ will be simply denoted with the symbol Φ. It is known from the literature on mathematical statistics, such as the work of SARKADI and VINCZE (1958) and PRÉKOPA (1962), that the number of broken jars at a specified loading force follows a binomial distribution with an expected value $= n\Phi$ and a standard deviation $= \sqrt{n\Phi(1-\Phi)}$,

If $n\Phi > 10$ this binomial distribution can be satisfactorily approximated by the normal distribution having the above expected value and deviation.

To every value of Φ and n a confidence interval can be assigned within which will lie with a given probability the number of broken jars from the total of n jars.

Let us denote the lower limit of the confidence interval with k_1 and the upper limit with k_2, then

$$k_1 < n\Phi < k_2 \tag{4}$$

If $n\Phi > 10$, then the binomial distribution can be approximated by the normal distribution, further if the values k_1 and k_2 are chosen symmetrically to the expected value $n\Phi$ then the lower and upper limit of the confidence interval pertaining to a probability of 95.4% can be calculated from the approximately valid equation

$$n\Phi - k_1 = k_2 - n\Phi \approx 2\sqrt{n\Phi(1-\Phi)}. \tag{5}$$

Division of the above equations by n leads to the lower and upper limits (k_1/n and k_2/n) of the interval of the relative frequency (k/n) pertaining to Φ and n as experimental result.

In the case when $n\Phi \leq 10$ the procedure is as follows: The cumulative distribution function of the binomial distribution

$$B_k = \binom{n}{k} \Phi^k (1-\Phi)^{n-k} \tag{6}$$

is calculated from $k = 0$ to a sufficiently high $k = m$ value. The k_1 and k_2 values are then sought at which the sum

$$\sum_{k_1}^{k_2} \binom{n}{k} \Phi^k (1-\Phi)^{n-k} \tag{7}$$

is equal to or just about greater than 0.954 and are in addition positioned most symmetrically of all the possible cases with respect to the value $n\Phi$. In this case it is in general impossible to find k_1 and k_2 values which are just symmetrical to the expected value. It is possible to find a sufficiently small value of Φ for a specified value of n, at which or under which $k_1 = 0$.

The relative frequencies pertaining to the limits of the interval are determined in the same way as in the case of $n\Phi > 10$.

In Tables 3, 4 and 5 the estimated values of the distribution functions Φ are given for the glass jar types and loading forces listed in Table 2, as well as the relative and absolute deviations of the frequency values — pertaining to the lower and upper limits of the confidence intervals of the number of breakings — from the values of the distribution function in case of samples with $n = 5000$ and $n = 10\,000$. The probability values assigned to the confidence intervals are also given; these are always equal to or greater than 95.4%.

It appears from the Tables that the greatest absolute deviations at $n = 5000$ and $n = 10\,000$, and at

$$x = 450 \text{ kp and } \Phi = 0.48 \text{ are:}$$

$$\Phi - \frac{k_1}{n} = \frac{k_2}{n} - \Phi = 1.413 \cdot 10^{-2} \text{ and } 0.999 \cdot 10^{-2}, \text{ resp.}$$

The pertaining relative deviations are 2.94% and 2.08%, resp.

These values were obtained for the 5/4 Comecon jars (Table 3) at a probability of 95.4%.

The greatest relative deviations at $n = 5000$ and $n = 10\,000$ at $x = 35$ kp and $\Phi = 1.10^{-4}$ are

$$\frac{k_2/n - \Phi}{\Phi} \cdot 100\% = 300\% \text{ and } 200\%, \text{ resp.}$$

again for Comecon jars, with probability values of 98.6% and 98.1%, resp., and absolute deviations of 3.10^{-4} and 2.10^{-4}, resp.

It appears further that small values of Φ are associated with high relative deviations, while the respective absolute deviations are small, and in case of high values of Φ the situation is exactly the reverse.

Thus, in order to be able to determine with adequate accuracy the distribution function at low loading forces, unfortunately, samples consisting of a great number of elements are needed. In fact not the number of elements is determined for a specified relative deviation, but the other way round: we must start from the largest sample size with which it is possible to perform the test in practice and then consider the reliability of the experimental results.

Table 3

The values of the confidence interval in the determination of the cumulative distribution function $\Phi(x|p = 0)$ of empty 5/4 Comecon type glass jars from preliminary experiments, $n = 5000$ and 10 000 jars

Loading force x, kp	Φ estimated	$\Phi - \frac{k_1}{n}$		$\frac{k_2}{n} - \Phi$		$\frac{\Phi - k_1/n}{\Phi} \cdot 100\%$		$\frac{k_2/n - \Phi}{\Phi} \cdot 100\,\%$		Probability level %	
		$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	$n=5000$	$n=10000$
35	0.00010	$0.010 \cdot 10^{-2}$	$0.010 \cdot 10^{-2}$	$0.030 \cdot 10^{-2}$	$0.020 \cdot 10^{-2}$	100	100	300	200	98.6	98.1
40	0.00058	$0.058 \cdot 10^{-2}$	$0.048 \cdot 10^{-2}$	$0.062 \cdot 10^{-2}$	$0.042 \cdot 10^{-2}$	100	82.75	106.90	72.41	97.1	96.2
45	0.00148	$0.108 \cdot 10^{-2}$	$0.077 \cdot 10^{-2}$	$0.092 \cdot 10^{-2}$	$0.077 \cdot 10^{-2}$	72.97	51.95	62.16	51.95	95.6	95.4
50	0.00200	$0.100 \cdot 10^{-2}$	$0.089 \cdot 10^{-2}$	$0.140 \cdot 10^{-2}$	$0.089 \cdot 10^{-2}$	50.00	44.68	70.00	44.68	95.6	95.4
100	0.00300	$0.155 \cdot 10^{-2}$	$0.109 \cdot 10^{-2}$	$0.155 \cdot 10^{-2}$	$0.109 \cdot 10^{-2}$	51.56	35.96	51.56	35.96		
150	0.00400	$0.179 \cdot 10^{-2}$	$0.126 \cdot 10^{-2}$	$0.179 \cdot 10^{-2}$	$0.126 \cdot 10^{-2}$	44.63	31.56	44.63	31.56		
200	0.00930	$0.272 \cdot 10^{-2}$	$0.192 \cdot 10^{-2}$	$0.272 \cdot 10^{-2}$	$0.192 \cdot 10^{-2}$	29.19	20.64	29.19	20.64		
250	0.06000	$0.672 \cdot 10^{-2}$	$0.475 \cdot 10^{-2}$	$0.672 \cdot 10^{-2}$	$0.475 \cdot 10^{-2}$	11.20	7.92	11.20	7.92		
300	0.13000	$0.951 \cdot 10^{-2}$	$0.673 \cdot 10^{-2}$	$0.951 \cdot 10^{-2}$	$0.673 \cdot 10^{-2}$	7.32	5.17	7.32	5.17	95.4	95.4
350	0.22000	$1.172 \cdot 10^{-2}$	$0.828 \cdot 10^{-2}$	$1.172 \cdot 10^{-2}$	$0.828 \cdot 10^{-2}$	5.33	3.77	5.33	3.77		
400	0.34000	$1.340 \cdot 10^{-2}$	$0.947 \cdot 10^{-2}$	$1.340 \cdot 10^{-2}$	$0.947 \cdot 10^{-2}$	3.94	2.79	3.94	2.79		
450	0.48000	$1.413 \cdot 10^{-2}$	$0.999 \cdot 10^{-2}$	$1.413 \cdot 10^{-2}$	$0.999 \cdot 10^{-2}$	2.94	2.08	2.94	2.08		

Table 4

The values of the confidence interval in the determination of the distribution function $\Phi(x|p=0)$ of empty 5/4 NHC type glass jars from preliminary experiments, n = 5000 and 10 000 jars

Loading force x, kp	Φ estimated	$\Phi - \frac{k_1}{n}$		$\frac{k_2}{n} - \Phi$		$\frac{\Phi - k_1/n}{\Phi} \cdot 100\%$		$\frac{k_2/n - \Phi}{\Phi} \cdot 100\%$		Probability level %	
		$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	$n=5000$	$n=10000$
35	0.00013	$0.013 \cdot 10^{-2}$	$0.013 \cdot 10^{-2}$	$0.027 \cdot 10^{-2}$	$0.017 \cdot 10^{-2}$	100	100	207.69	130.77	97.2	95.7
40	0.00070	$0.070 \cdot 10^{-2}$	$0.050 \cdot 10^{-2}$	$0.070 \cdot 10^{-2}$	$0.050 \cdot 10^{-2}$	100	71.43	100	71.43	97.3	96.6
45	0.00313	$0.158 \cdot 10^{-2}$	$0.112 \cdot 10^{-2}$	$0.158 \cdot 10^{-2}$	$0.112 \cdot 10^{-2}$	50.48	35.69	50.48	35.69		
50	0.00500	$0.200 \cdot 10^{-2}$	$0.141 \cdot 10^{-2}$	$0.200 \cdot 10^{-2}$	$0.141 \cdot 10^{-2}$	39.90	28.21	39.90	28.21		
100	0.01500	$0.344 \cdot 10^{-2}$	$0.243 \cdot 10^{-2}$	$0.344 \cdot 10^{-2}$	$0.243 \cdot 10^{-2}$	22.92	16.21	22.92	16.21		
150	0.03000	$0.483 \cdot 10^{-2}$	$0.341 \cdot 10^{-2}$	$0.483 \cdot 10^{-2}$	$0.341 \cdot 10^{-2}$	16.08	11.37	16.08	11.37	95.4	95.4
200	0.05500	$0.645 \cdot 10^{-2}$	$0.456 \cdot 10^{-2}$	$0.645 \cdot 10^{-2}$	$0.456 \cdot 10^{-2}$	11.72	8.29	11.72	8.29		
250	0.17000	$1.062 \cdot 10^{-2}$	$0.751 \cdot 10^{-2}$	$1.062 \cdot 10^{-2}$	$0.751 \cdot 10^{-2}$	6.25	4.42	6.25	4.42		
300	0.28500	$1.277 \cdot 10^{-2}$	$0.903 \cdot 10^{-2}$	$1.277 \cdot 10^{-2}$	$0.903 \cdot 10^{-2}$	4.48	3.17	4.48	3.17		
350	0.42500	$1.398 \cdot 10^{-2}$	$0.989 \cdot 10^{-2}$	$1.398 \cdot 10^{-2}$	$0.989 \cdot 10^{-2}$	3.29	2.33	3.29	2.33		
400	0.57000	$1.400 \cdot 10^{-2}$	$0.990 \cdot 10^{-2}$	$1.400 \cdot 10^{-2}$	$0.990 \cdot 10^{-2}$	2.46	1.74	2.46	1.74		

Table 5

The values of the confidence interval in the determination of the distribution function $\Phi(x|p=0)$ of empty 5/1 N type glass jars from preliminary experiments, n = 5000 and 10 000 jars

Loading force x, kp	Φ estimated	$\Phi - \frac{k_1}{n}$		$\frac{k_2}{n} - \Phi$		$\frac{\Phi - k_{1:}/n}{\Phi} \cdot 100\%$		$\frac{k_2/n - \Phi}{\Phi} \cdot 100\%$		Probability level %	
		$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	$n = 5000$	$n = 10000$	n=5000	n=10000
110	0.001	$0.080 \cdot 10^{-2}$	$0.060 \cdot 10^{-2}$	$0.080 \cdot 10^{-2}$	$0.060 \cdot 10^{-2}$	80.00	60.00	80.00	60.00	96.2	96.3
120	0.003	$0.155 \cdot 10^{-2}$	$0.109 \cdot 10^{-2}$	$0.155 \cdot 10^{-2}$	$0.109 \cdot 10^{-2}$	51.56	36.46	51.56	36.46		
130	0.004	$0.179 \cdot 10^{-2}$	$0.126 \cdot 10^{-2}$	$0.179 \cdot 10^{-2}$	$0.126 \cdot 10^{-2}$	44.63	31.56	44.63	31.56		
140	0.006	$0.218 \cdot 10^{-2}$	$0.154 \cdot 10^{-2}$	$0.218 \cdot 10^{-2}$	$0.154 \cdot 10^{-2}$	36.40	25.74	36.40	25.74		
150	0.008	$0.252 \cdot 10^{-2}$	$0.178 \cdot 10^{-2}$	$0.252 \cdot 10^{-2}$	$0.178 \cdot 10^{-2}$	31.50	22.27	31.50	22.27		
200	0.010	$0.281 \cdot 10^{-2}$	$0.199 \cdot 10^{-2}$	$0.281 \cdot 10^{-2}$	$0.199 \cdot 10^{-2}$	28.14	19.89	28.14	19.89		
250	0.020	$0.396 \cdot 10^{-2}$	$0.280 \cdot 10^{-2}$	$0.396 \cdot 10^{-2}$	$0.280 \cdot 10^{-2}$	19.80	14.00	19.80	14.00	95.4	95.4
300	0.050	$0.616 \cdot 10^{-2}$	$0.436 \cdot 10^{-2}$	$0.616 \cdot 10^{-2}$	$0.436 \cdot 10^{-2}$	12.33	8.72	12.33	8.72		
350	0.080	$0.767 \cdot 10^{-2}$	$0.543 \cdot 10^{-2}$	$0.767 \cdot 10^{-2}$	$0.543 \cdot 10^{-2}$	9.59	6.78	9.59	6.78		
400	0.110	$0.885 \cdot 10^{-2}$	$0.626 \cdot 10^{-2}$	$0.885 \cdot 10^{-2}$	$0.626 \cdot 10^{-2}$	8.05	5.69	8.05	5.69		
450	0.140	$0.981 \cdot 10^{-2}$	$0.694 \cdot 10^{-2}$	$0.981 \cdot 10^{-2}$	$0.694 \cdot 10^{-2}$	7.01	4.96	7.01	4.96		
500	0.180	$1.087 \cdot 10^{-2}$	$0.768 \cdot 10^{-2}$	$1.087 \cdot 10^{-2}$	$0.768 \cdot 10^{-2}$	6.04	4.27	6.04	4.27		
550	0.210	$1.152 \cdot 10^{-2}$	$0.815 \cdot 10^{-2}$	$1.152 \cdot 10^{-2}$	$0.815 \cdot 10^{-2}$	5.49	3.88	5.49	3.88		
600	0.250	$1.225 \cdot 10^{-2}$	$0.866 \cdot 10^{-2}$	$1.225 \cdot 10^{-2}$	$0.866 \cdot 10^{-2}$	4.90	3.46	4.90	3.46		
650	0.290	$1.283 \cdot 10^{-2}$	$0.907 \cdot 10^{-2}$	$1.283 \cdot 10^{-2}$	$0.907 \cdot 10^{-2}$	4.43	3.13	4.43	3.13		
700	0.330	$1.330 \cdot 10^{-2}$	$0.940 \cdot 10^{-2}$	$1.330 \cdot 10^{-2}$	$0.940 \cdot 10^{-2}$	4.03	2.85	4.03	2.85		
750	0.370	$1.366 \cdot 10^{-2}$	$0.965 \cdot 10^{-2}$	$1.366 \cdot 10^{-2}$	$0.965 \cdot 10^{-2}$	3.69	2.61	3.69	2.61		
800	0.420	$1.396 \cdot 10^{-2}$	$0.987 \cdot 10^{-2}$	$1.396 \cdot 10^{-2}$	$0.987 \cdot 10^{-2}$	3.32	2.35	3.32	2.35		

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Symbols

B_k = binomial distribution according to Eq. (6) Part II

$F(x_0, N)$, $F(x_0, N \to \infty)$, $F(x_0, \infty)$ = cumulative distribution function giving the average breaking ratio by accounting for the step-wise breaking process

$F(x_{0CR}, \infty)$ = value of $F(x_0, \infty)$ at $x_0 = x_{0CR}$

$F(x_{0K}, N)$ = values of $F(x_0, N)$ at $x_0 = x_0K$ values $(1 \leq K \leq R)$

H = number of useful numbers generated by the program described in Part I

j = serial number in the subscript indicating the successive steps in the step-wise breaking process

k, k_1, k_2 = number of broken jars in a sample consisting of n elements at a given load in relation to the binomial distribution according to Eq. (6), Part II

K = serial number in the subscript indicating the layers in the stacks placed one on top of the other counted from the bottom upwards according to the relationship (1) in Part II $(1 \leq K \leq R)$

K = total number of numbers generated by the program described in para 3, Part I

l = serial number in Eq. (10), Part I $(l = 0, 1, 2 \ldots M_B)$

L = number of jars in a set consisting of a finite number of elements which remain undamaged even under the maximum load occurring in course of the step-wise breaking process

m = the specified value of k in connection with the binomial distribution according to Eq. (6), Part II

m_j = number of jars broken in the j-th step

m_r = number of jars broken in the last step of the step-wise breaking process

M = total number of jars broken in course of the step-wise breaking process

M_j = number of jars from a set consisting of a finite number of elements which break in course of the step-wise breaking process when the load is between x_j and x_{j-1}

M_B = the maximum total number of jars still permissible from the aspect of safety, broken in the step-wise breaking process

n = number of elements in the sample

N = initial number of jars in the layer (without breakings)

p = specified value of the vacuum in the jar (kp/cm²)

p_i = given value of the vacuum in the jar as a probability variable (kp/cm²)

$p_{\max}$ = the highest possible value of vacuum in the jar (kp/cm²)

P = probability of the occurrence of a definite step-wise breaking process

$P(M > M_B)$ = probability of a higher number of jars being broken in course of the step-wise breaking process than the specified M_B value

$P(M \leq M_B)$ = probability of fewer or the same number of jars being broken in course of the step-wise breaking process than the specified M_B value

P_M = probability of M jars being broken (independently of the order of breakings) in course of the step-wise breaking process

R = the number of layers in the stack column
U = number of jars in a set consisting of a finite number of jars
$[U]$ = set consisting of an infinite number of jars
x = the load on a given jar (kp)
$\overline{x}$ = expected value of the sample distribution in Table 1, Part II (kp)
x_e = limit value of the average load in the step-wise breaking process of layers with infinite number of elements (kp)
x_{eCR} = average load pertaining to the critical primary load (kp)
x_h = minimum breaking strength (kp)
x_i = breaking force characteristic of the glass jar, *i.e.* breaking strength as a probability variable (kp)
x_j = loading force in the j-th step (kp)
x_0 = primary load, or the average load in step 0 (kp)
x_{0K} = primary load in the K-th layer (kp)
x_{0CR} = critical primary load (kp)
x_m = maximum breaking strength (kp)
y_i = breaking force (breaking strength) as a probability variable for vacuum-free jars (kp)
Z = average breaking ratio (percentage) according to Eq. (1) Part II
$\varphi(x)$ = density function pertaining to the breaking force x_i as a probability variable
$\varphi(x|p)$ = conditional probability density function pertaining to the density function $\varphi(x)$ when the value of p is constant
$\Phi(x)$ = cumulative distribution function pertaining to the breaking force x_i as a probability variable
$\Phi(x|p = \text{constant})$ = conditional cumulative distribution function pertaining to the distribution function $\Phi(x)$ when the value of p is constant
$\Phi(x|p = 0)$ = conditional cumulative distribution function pertaining to the distribution function $\Phi(x)$ when the value of p is zero
$\psi(p)$ = density function pertaining to a vacuum of the value of p_i as a probability variable
$\Psi(p)$ = cumulative distribution function of the probability variable p_i

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NEW STARCHES: THE PROPERTIES OF THE STARCH FROM *AMARANTHUS PANICULATUS*, LINN.

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Starch with circular granules of 3.2 μm size was isolated from rajgeera (*Amaranthus paniculatus*, Linn.). The starch was waxy, with negligible iodine affinity. It had a higher gelatinization temperature and was more sensitive to disruption during heating, when compared to corn starch.

Rajgeera seeds are obtained from the plant (*Amaranthus paniculatus*, Linn.), an Asiatic species, cultivated throughout India. The tender shoots and leaves are used as a leafy vegetable while the seeds furnish "anardana", a food grain very popularly consumed in India in the form of sweets like *chikki*, *laddu* which are especially consumed on fasting days. The seed contains around 13–16% protein, 55–60% carbohydrate and is a good source of calcium, phosphorus and iron (WEALTH OF INDIA, 1952). In our search for new indigenous sources of starch, starch was isolated from this seed and was studied for the microscopic characteristics, solubility in water, iodine affinity, gelatinization range, viscosity profile at various temperatures and digestibility (MODI & KULKARNI, 1976) in order to find out the possibility of substituting this new starch for conventional starch like corn for use in various food preparations.

1. Materials and methods

1.1. Preparation of starch

Starch was isolated from cleaned seeds of rajgeera (*Amaranthus paniculatus*, Linn.) by using the alkali steeping method (SARIN & QURESHI, 1938). After washing it properly, it was dried at room temperature and then at 50 °C overnight.

1.2. Determinations

Starch obtained as above was analysed for the moisture, protein, fat, fibre and ash contents by standard AOAC methods (AOAC, 1960).

Amylose and amylopectin contents were determined colorimetrically (MCCREADY & HASSID, 1943).

Solubility of starch in water was determined as described by KERR (1950).

Iodine affinity was determined by potentiometric titration according to SCHOCH (1964). Gelatinization temperature was determined by the method of MACMASTERS (1964).

Brabender pasting temperature and maximum gelatinization viscosity were determined using *Brabender Amylograph* viscometer. For this 9.3 parts of starch solids per 100 parts of water were made to a total volume of 530 ml (SMITH, 1964).

Standard corn starch obtained from MESSRS. CORN PRODUCTS CO. (INDIA) PVT. LTD., was used for comparison.

2. Results and discussion

Average values of three determinations on the proximate composition of rajgeera starch were: moisture: 13.1 ± 0.529; protein: 0.57% ± 0.0264; fat: 0.38% ± 0.02; fibre: 0.5% ± 0.01 and ash: 0.8% ± 0.0529.

Table 1 shows data on amylose, amylopectin content, solubility, iodine affinity and gelatinization temperature.

The basic difference between the corn and rajgeera starch as shown in Table 1, *viz.*, the latter is a waxy starch, was found to influence the properties studied.

Table 1

General properties of corn and rajgeera starch

Starch	Microscopic characteristics		Amylose %	Amylopectin %	Solubility at 30 °C %	Iodine affinity %	Gelatinization range °C
	Size	Shape					
Corn	13.54 μm to 13.90 μm	round	23.5 ± 0.264	76.5 ± 0.264	0.4 ± 0.0264	3.1 ± 0.1	57 – 68
Rajgeera	3.2 μm	circular	–	100	1.6 ± 0.02	0.0	63 – 74

Total number of determinations in each case was 3

For example, in the present case, solubility of rajgeera starch was 1.6% as against 0.4% for corn which was in agreement with the fact that the more the amylopectin content the more soluble is the starch. Absence of amylose in rajgeera starch also gave negligible iodine affinity values as against corn which showed 3.11%. Presence of bulky amylopectin molecule in rajgeera tending to resist initial gelatinization was responsible in giving a higher initial gelatinization temperature of 63 °C as against corn which had 57 °C (SCHOCH & MAYWALD, 1956). The gelatinization range was 63–74 °C for rajgeera and 57–68 °C for corn. Small sized granules are known to gelatinize at higher temperatures (MULLEN & PACSU, 1942; COLLISON *et al.*, 1960) which is also found to be true of rajgeera starch having granule size of 3.2 μm.

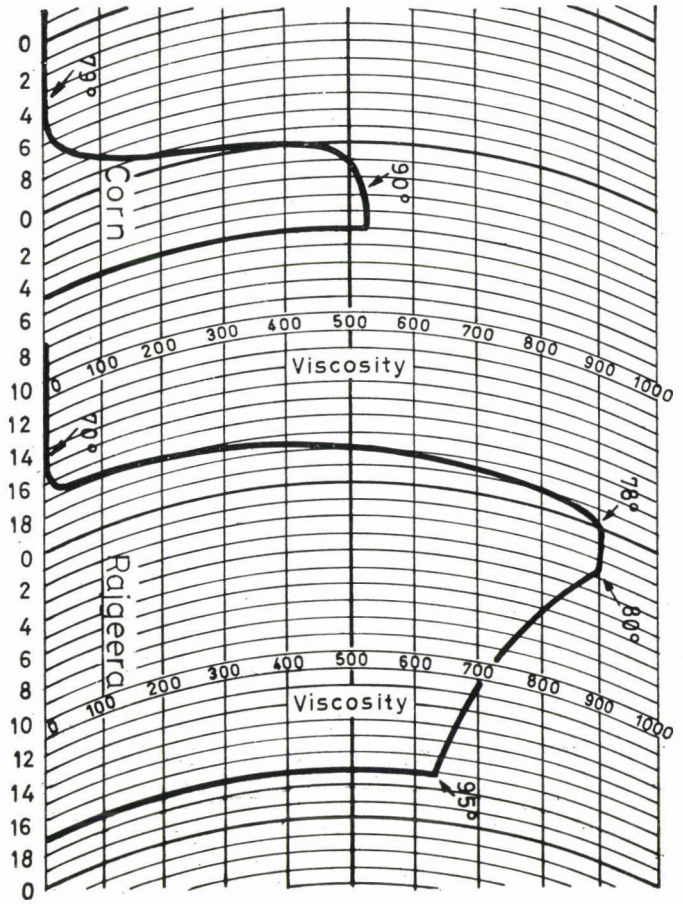

Fig. 1. Amylograms of corn and rajgeera starch

Brabender amylograms of the the two starches are as shown in Fig. 1.

Examination of the curves shows that for rajgeera, peak viscosity of 900 BU could be obtained at a temperature of 78 °C and the minimum pasting temperature was 70 °C, comparing well with that reported for *Amaranthus retroflexus* (Rao & Goering, 1970). Corn on the other hand, reached peak viscosity of 520 BU at 90 °C and had minimum pasting temperature of 79 °C. Since in the present case, it was not possible to follow the change in viscosity during subsequent cooling, the viscosity change was followed up to the peak value followed by additional heating to 95 °C. On comparison of the corn and rajgeera starch under these additional heating conditions, the peak viscosity of 900 BU for rajgeera remained constant from 78 ° to 80 ° and dropped down from 900 BU to 640 BU till it reached 95 °C. In corn, however, it was maintained at 520 BU from 90° even up to 95 °C. The high peak viscosity obtained

with rajgeera starch is indicative of its high swelling power because of the amylopectin constituent exclusively present in this starch. The fall in viscosity on further heating appears to be a result of easy breakdown of the fragile amylopectin molecules. On the other hand, the corn starch containing amylose showed a steady rise which was maintained even after heating to a higher temperature of 95 °C. From these observations, it appears that rajgeera starch is better than corn starch in its application as far as preparation of pastes of higher viscosity by mild heating is concerned since it gives more viscous paste at a lower temperature; however, it will yield a minimum setback on cooling as is true of other waxy starches (FREEMAN & VERR, 1972).

*

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A STUDY OF TOBACCO LIPIDS

PART I. — CHANGES IN THE LIPIDS OF TOBACCO LEAVES IN THE COURSE OF VEGETATIVE DEVELOPMENT OF THE PLANT

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The vegetative development of the tobacco plant was studied by chemical analysis of the lipids. Two local varieties, *Virginia* and *Burley* plants were chosen as model plants. After planting the seedlings, we took samples in 9 different phenophases of the plants over a period of 16 weeks. The tobacco leaves were dried and in a two-step process of extraction, neutral lipids (triglycerids, wax esters, *etc.*) and polar lipids (phospholipids, glycolipids, *etc.*) were obtained. According to our measurements, the total lipid content of tobacco leaves varied between 8–20% and showed characteristic values at different stages of development (planting of the seedlings, intensive growth period, blossoming, technological ripeness). Based on the above, examination of the lipid content may offer a method for the determination of technological ripeness, all the more so, as in our experiments, maximum lipid content was detected at the final stage of development.

Several varieties of tobacco (*Nicotiana tabacum* L.) are grown in Hungary. Breaking of the technologically ripe leaves proceeds gradually from the lower senescent (yellow) leaves to the upper leaves and finally, within a period of approximately one month, the whole foliage is harvested.

Determination of the technological ripeness of leaves is based mainly on the subjective judgement of the grower. KANDRA (1972) from the INSTITUTE FOR TOBACCO RESEARCH (Debrecen) studied the change in the composition of the leaves at different node levels and at various stages of development for *Virginia* leaves. In senescent but technologically ripe leaves he observed an increase in the amount of reducing sugar content and phenolic compounds (resins) and a decrease in the chlorophyl content. Since the fatty materials of the leaves have not been studied, we tried to follow the vegetative development of the tobacco plant by chemical analysis of the lipids. Very few papers were found on the subject and only two publications. MOSELEY and co-workers (1951) and BACOT (1960) gave data on the resin and wax content of tobacco leaves measured at various node levels of the plant.

Of the chemical components of the tobacco leaf, fatty substances have seldom been chosen so far, for the subject of study since they were not considered very important regarding industrial production. Alkaloids, and especially nicotine which is responsible for some of the physiological symptoms caused by smoking, are considered perhaps the most important components of the tobacco leaf. The flavour of smoke is also greatly affected by simple (reducing) sugars as well as other organic nitrogen compounds. Considering the fact,

however, that the presence of even small amounts of lipids may highly enhance organoleptic effects, the taste and flavour of smoke, we presume that the results of investigations in the field of lipid chemistry may also offer important data for the technology of tobacco processing.

Most data on tobacco lipids can be traced by analysis of the tobacco smoke. According to KITAMURA (1971) and HIGMAN (1974) aldehydes, ketones and acids are formed from triglycerides, as the products of pyrolysis, in the course of oxidative decomposition. As stated by SCHMELTZ (1969) and RUDENKO (1969) monoaromatic hydrocarbons and benzpyrene may also be formed from unsaturated fatty acids (linoleic and linolenic acids) by way of ring closure and decarboxylation. The studies of SCHLOTZHAUER (1970), BROWN (1971) and HENDERSON (1968) showed mainly paraffin hydrocarbons of carbon number 27–31 to yield cyclic hydrocarbons and mono-olefines as the pyrolysis products in tobacco smoke. KLIMISCH and STADLER (1972) detected pyrolysis products derived from plant sterols. SCHUMACHER and VESTAL (1974) determined derivatives from tri- and diterpenes and lactones from fatty acids.

For the determination of possible differences in the lipid content of various tobacco varieties, we have chosen *Virginia* and *Burley* tobaccos as varieties grown in largest amounts with most variable internal and external qualities. According to FAO statistics, *Virginia* tobacco constitutes 43% of world production in tobacco, while the *Burley* variety, with its favourable properties of production and yield (22–24 leaves), is also gaining importance all over the world.

A marked difference can be observed in the development of the two varieties. *Virginia* tobacco grows gradually while variety *Burley* shows first no development and shoots up only later, quickly surpassing variety *Virginia* (MÓGER & SZÜCS, 1966). The rate of plant development can be followed by measuring the height of the plant and the number of leaves. Numerical values for the varieties *Burley* are given by KAWATOKO and co-workers (1973). Leaf samples of varieties *Burley* Nos. 21 and 49 were taken every 10 days. Measurement data are shown in the diagram in Fig. 1.

An attempt has been made to compare values obtained by KANDRA (1972) for *Virginia Krakowska* tobacco in a similar diagram, presented in Fig. 2.

Comparison of the two diagrams shows that there is a numerically evaluable difference in the vegetative development of the two varieties. In order to determine the difference in the rate of development of the *Virginia* and *Burley* varieties, therefore, it seemed feasible to measure the change in stem-height and compare these data with the results of our investigations in lipid chemistry.

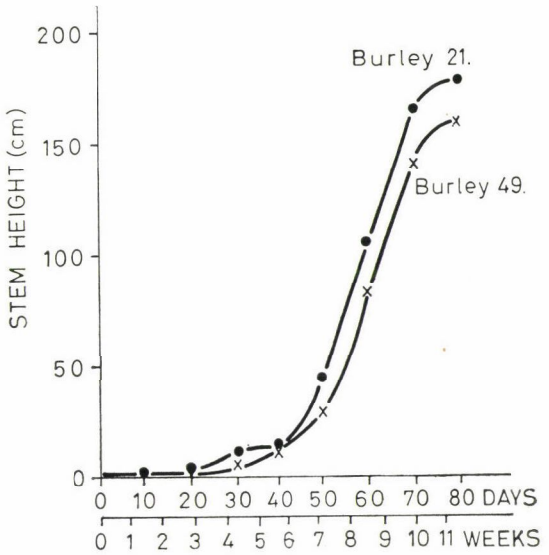

Fig. 1. Stem height as a function of time for *Burley* 21 and 49 varieties based on data given by KAWATOKO and co-workers (1973)

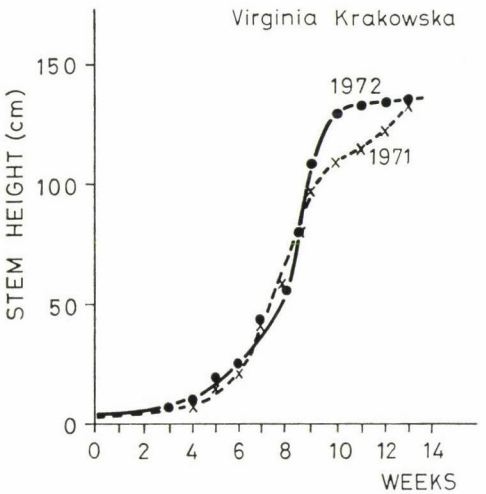

Fig. 2. Stem height as a function of time for *Virginia Krakowska* tobacco based on data given by KANDRA (1972)

1. Materials and methods

The samples of different tobacco varieties were collected from fields of the ÉRD EXPERIMENTAL STATION OF THE INSTITUTE FOR TOBACCO RESEARCH. On the average we have taken 20 samples of greenhouse seedlings. After planting the seedlings in the field, leaf samples were taken of each of the 25

stems, every 14th day from the end of May 1974 up to the end of September. Tobacco leaf samples were prepared by an industrial procedure described by GÄRTNER (1944) and DIETZE (1953). The varieties studied were locally grown *Virginia Delcrest* and *Burley* 48.

Soil and environmental conditions. The soil in the fields of Érd Station is an adobe-type chernosyom with adsorption number 38–40 according to *Arany*. The soil is poor in nitrogen, medium in phosphorus and its medium content of potassium salt is characteristic. Considering climatic conditions, the area shows signs of aridity with low annual rainfall. The average temperature is suitable for growing tobacco. The 1974 parameters are given in Table 1 compared with average values of parameters for the whole country in the period 1900–1950.

Table 1
Plant production parameters of the experiments in 1974 (Érd) compared with the averages for 1900–1950

Rainfall (mm)

Month	IV.	V.	VI.	VII.	VIII.	IX.	Total
1974	40	61	38	36	64	70	309
1900–1950	52	69	67	50	48	45	331
Deviation ±	−12	−8	−26	−14	+16	+25	−22

Temperature: (°C)

Month	IV.	V.	VI.	VII.	VIII.	IX.	Total*
1974	9.8	13.9	16.6	19.5	22.0	16.0	97.8
1900–1950	11.4	16.8	19.9	21.9	21.1	16.9	108.0
Deviation ±	−1.6	−2.9	−3.3	−2.4	+0.9	−.09	−10.2

* Total temperature = sum of the monthly average temperatures

Relative humidity: (%)

Month	V.	VI.	VII.	VIII.	IX.
Average values	84	79	66	72	78

Extraction of lipids. After breaking, the tobacco leaves were dried and powdered. The tobacco powder (15–20 g) was extracted with petroleum ether (b.p. 40–70 °C) in a *Soxhlet*-apparatus for 8 hours by the method of KAUFMANN (1958). The solvent was distilled off and the lipid weight was measured and

related to the amount of air-dry material. After extraction the tobacco powder was dried in air and extracted in a second step with a 2 : 1 v/v mixture of chloroform–methanol for further 8 hours by the method of KATES (1972). According to MCKILLICAN and LAROSE (1974) by the two-step extraction procedure both free and tissue-bound lipids are obtained separately. The first extract contained mainly neutral lipids (triglycerides, wax esters, hydrocarbons), while the second extract gave tissue-bound forms of triglycerides and fatty acids as well as polar lipids (phospholipids) and plant pigments. Distribution of the lipid groups in both fractions has been examined by thin-layer chromatography.

Thin-layer chromatography. Neutral lipids in the petroleum ether extract were analysed by chromatography according to MANGOLD and MALINS (1960) and the polar lipids of the chloroform-methanol extract were determined by MARINETTI's (1967) method. Samples (250–500 micrograms) were applied on a thin-layer plate and *Merck* standard reagents were used for identification. Polar lipids were detected using specific reagents according to KATES (1972): 1. ammonium molibdenate–perchloric acid for phosphatides; 2. ninhydrin for amino-phosphatide; 3. the *Dragendorff*-reagent for the cholin-group; 4. orcin-sulphuric acid for substances with carbohydrate-content and 5. a saturated solution of antimony trichloride for sterols according to WOOLEN and co-workers (1972).

Saponification. For quantitative analysis, a fraction of the petroleum ether extract was decomposed by alkaline hydrolysis into fatty acids (soap) and neutral fractions. The non-saponifiable (neutral) fraction was separated from the saponified fatty acids by extraction with a 1 : 1 v/v mixture of petroleum ether–diethyl ether. After extraction the fatty acids were liberated from the soap with hydrochloric acid at pH = 2 and extracted with the same mixture. Both extracts were distilled and weighed, then the fatty acids converted into methyl esters for gas chromatography by STAHL's method (1969). The change in the weight ratio related to the two fractions obtained by the above method was determined at different phenophases.

Gas chromatographic determination of fatty acids. The distribution of fatty acid methyl esters was carried out by different gas chromatographic measurements. Distribution according to the number of unsaturated bonds for components containing 16–18 carbon atoms was measured at a 10% DEGS (diethylene glycol succinate) stationary phase under isothermic conditions at 185 °C, while distribution according to chain length for fatty acids with 12–30 carbon atoms was determined on 1% OV-101 (methyl silicone) as the stationary phase in the range of 100–315 °C with temperature programmed instrument. Qualitative evaluation was made using standard fatty acid methyl ester mixtures and quantitative determination was performed by measuring the peak areas of the chromatogram.

2. Results

Graphical demonstration of plant development. The curve showing the development of *Virginia* and *Burley* varieties grown under conditions at Érd, reflects the differences outlined in the introduction. For 6 weeks after planting greenhouse seedlings in the field no significant growth could be observed in stem height. This period has therefore been named as the initial phase of development. Intensive growth for variety *Virginia* started at the 6th week while in case of variety *Burley* it started at the 8th week, rapidly increased after a

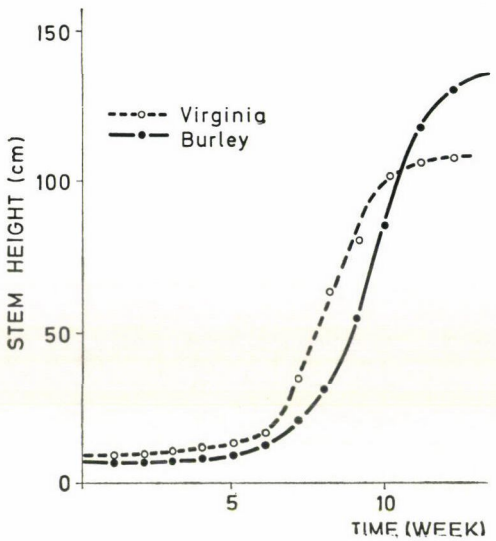

Fig. 3. Stem height as a function of time for the varieties *Virginia Delcrest* and *Burley* 48. grown in the course of the present experiment at the Érd Experimental Station of the Institute for Tobacco Research. Plotted values are the average data measured on 25 plants each

transitional phase. This phase of rapid development lasted until the 10th week for *Virginia* and till the 12th week for *Burley* tobacco. Following this, during the final phase of development no significant growth could be observed. (Fig. 3).

2.1. Quantitative changes in the lipid content at different phases of vegetative development

The different phases determined by the curve of vegetative development (initial stage of development, intensive growth and final stage of development) showed certain deviations in the results of lipid content and composition measurements.

2.1.1. Effect of planting tobacco seedlings on the lipid content of the plant. Extraction of the lipids of *Virginia Delcrest* and *Burley* 48 seedlings resulted in

considerably different data for samples taken from the greenhouse and from the field already two weeks after planting.

The amount of petroleum ether and chloroform–methanol extracts of the seedlings remaining in the greenhouse showed nearly half as much decrease as found in seedlings planted in the field. The total amount of lipids was calculated from the sum of average amount of lipids extracted by the two-step process.

The lipid content of varieties *Virginia* and *Burley* showed marked differences only in petroleum ether extracts.

Table 2

Lipid content of tobacco seedlings related to air-dry material

Sample	$\bar{P}$	s_P	$\bar{C}$	s_C	T
Virginia					
greenhouse 0 week	3.2	0.20	11.5	0.36	14.7
greenhouse 2nd week	2.7	0.17	8.3	0.36	11.0
field 2nd week	2.1	0.20	6.8	0.17	8.9
Burley					
greenhouse 0 week	2.3	0.20	10.0	0.36	12.3
greenhouse 2nd week	2.1	0.26	8.2	0.26	10.3
field 2nd week	1.1	0.17	7.3	0.35	8.4

$\bar{P}$ = average value of petroleum ether extracts; s_P = standard deviation (related to P); $\bar{C}$ = average value of chloroform-methanol extracts; s_C = standard deviation (related to C); T = total lipid content ($\bar{P} + \bar{C}$). Values obtained from 3 parallel measurements

2.1.2. Change in the lipid content in the initial phase of development. The change in the lipid content of the two tobacco varieties in the period 0–8th week is demonstrated in Fig. 4.

The lipid content of leaves of field plants between the 2nd and 8th weeks varied according to the variety and solvent. In the case of *Virginia* tobacco the amount of both extracts showed a nearly steady increase while with variety *Burley* sudden increase in the amount of petroleum ether extract could be observed during the 2nd to 4th weeks. In the case of the chloroform–methanol extract on the other hand, only a slight decrease could be detected. The increase in the lipid content of variety *Burley* can be considered constant only after the 4th week.

2.1.3. Study of the intensive development phase. After extraction by the two-step process the lipid content of the two varieties was summed up and the weights of the fractions obtained during the complete period of vegetative development are plotted in Fig. 5.

The amount of both petroleum ether and chloroform–methanol extracts

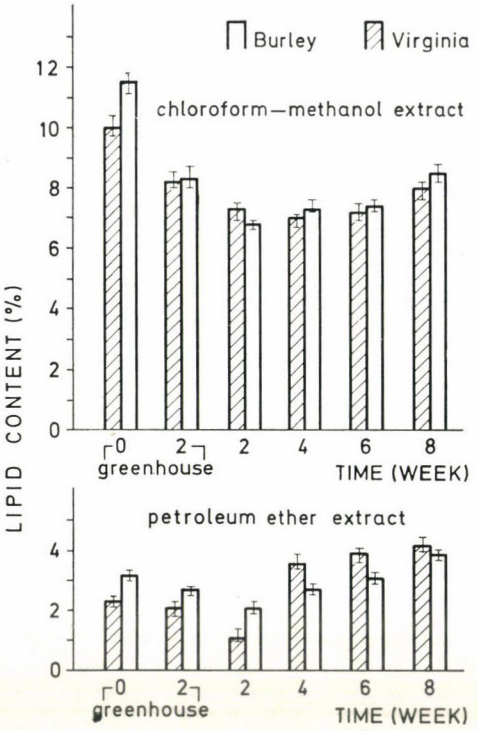

Fig. 4. Changes in the lipid content of the leaves in the initial phase of growth of the tobacco plant. Extraction of tobacco samples was carried out in two successive steps: (1) with petroleum ether and (2) with chloroform–methanol. Extract weights related to air-dry material (%). Plotted values are the averages of 3 measurements ($n = 3$). Deviations (maximum–minimum) shown by vertical lines. Samples, unless not marked otherwise, were taken from the field

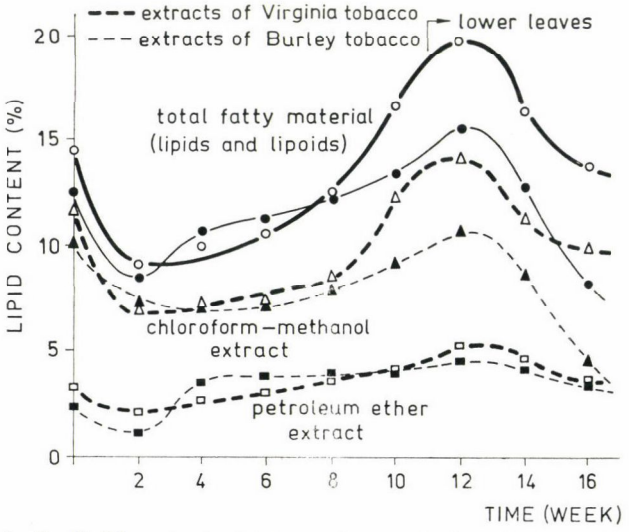

Fig. 5. Changes in the lipid content of tobacco leaves during plant growth up to 16 weeks (data shown from 12th to 16th weeks indicate values of the lower leaves). Signs: –□– petroleum ether extract; –△– chloroform methanol extract; –○– sum of the two extract weights related to air-dry material (%); open signs: *Virginia*, full signs: *Burley*. Plotted values are the averages of 3 measurements ($n = 3$)

has shown a rapid increase in the phase of intensive development. The increase is especially significant in the chloroform–methanol extract containing polar lipids (phospholipids, glycolipids) and plant pigments. The increase in lipid content is much more intensive in this phase for variety *Virginia* than for *Burley* tobacco.

2.1.4. Changes in the lipid content in the final phase of development. With the decrease in the rate of vegetative development from the 10th to the 12th week there is a well perceptible difference in the lower and upper leaves of the tobacco plant, therefore lipid content determinations have been carried out separately. The lower (bottom) leaves have reached the stage of technological ripeness, suitable for breaking, therefore these values have been depicted in Fig. 5. The difference in the lipid content of lower and upper leaves is shown in Table 3.

Table 3

Lipid content of the lower (bottom) and upper (top) leaves in the final stage of development (12–16 weeks)

Virginia

	$\bar{P}$	s_P	$\bar{C}$	s_C	T
Lower leaves					
12th week	5.5	0.36	14.1	0.36	19.6
14th week	4.8	0.27	11.4	0.44	16.2
16th week	3.7	0.35	10.4	0.46	14.1
Upper leaves					
12th week	2.2	0.27	5.0	0.40	7.2
14th week	4.5	0.35	10.9	0.46	15.4
16th week	3.1	0.36	11.7	0.52	14.8

Burley

	$\bar{P}$	s_P	$\bar{C}$	s_C	T
Lower leaves					
12th week	4.8	0.27	10.8	0.50	15.6
14th week	4.1	0.35	8.9	0.46	13.0
16th week	3.6	0.73	4.4	0.52	8.0
Upper leaves					
12th week	3.1	0.30	7.8	0.44	10.9
14th week	4.4	0.36	14.2	0.56	18.6
16th week	4.6	0.40	9.9	0.35	14.5

$\bar{P}$ = average value of petroleum ether extracts; s_P = standard deviation (related to P); $\bar{C}$ = average value of chloroform–methanol extracts; s_C = standard deviation (related to C); T = total lipid content ($\bar{P} + \bar{C}$). Values obtained from 3 parallel measurements

In examining the amount of petroleum ether and chloroform–methanol extracts we found maximum lipid content in the lower leaves in the 12th week, while the upper fresh shoots showed considerably lower amount of lipids. After this period, the lipid content of the lower, senescent leaves rapidly decreased. Comparison of the two tobacco varieties showed the lipid content in variety *Burley* to be lower and to decrease more rapidly mainly in its chloroform–methanol extract. The lipid content of the upper leaves increased even after the 12th week and reached a maximum value at the 14–15th week.

2.2. Changes in the fatty acid fraction of the petroleum ether extract in various phases of development

After extraction with petroleum ether, the less bound neutral lipids (triglycerides, wax esters) were separated by alkaline hydrolysis into a fatty acid and an unsaponifiable fraction. The ratio of fatty acid fraction during the entire vegetative development is presented in Fig. 6.

There is no difference in the ratio of fatty acid fractions in seedlings of the two varieties. The ratio of fatty acid fraction shows a constant rapid decrease with both varieties in the initial phase in field plants. It reaches minimum value in variety *Virginia* in the 6th week and in *Burley* tobacco in the 8th

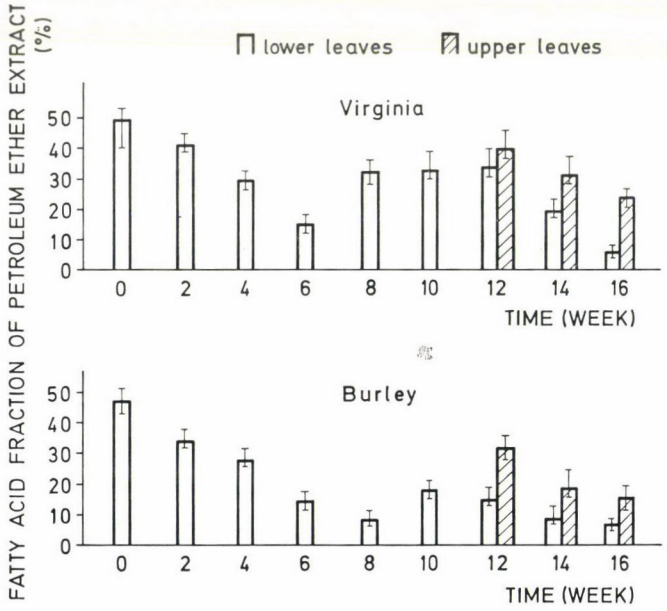

Fig. 6. Changes in the fatty acid fraction in the petroleum ether extract in the course of plant growth (up to 16 weeks). The data reflect the ratio of the fatty acid fraction obtained by saponification of the petroleum ether extract (%). Plotted values are the averages of 3 measurements ($n = 3$). Vertical lines illustrate extreme values. The results obtained in the period between the 12th and 16th weeks are shown separately for lower leaves (blank columns and for upper leaves (lined columns)

week. In the phase of intensive development the ratio of fatty acid fraction increases although in the case of *Burley* plant it does not reach the value obtained with variety *Virginia*. In the final phase of development both varieties display a certain decrease. The ratio of fatty acid fraction in the petroleum ether extract is throughout higher in the top (upper) shoots than in the bottom (lower) leaves.

2.3. Qualitative investigation of the fatty acid fraction of the petroleum ether extract by gas chomatography

Distribution of the C_{14}–C_{18} fatty acid components according to the number of unsaturated bonds was determined by gas chromatography under isothermic conditions in the form of methyl esters. Fatty acid composition of lipids in tobacco seeds, greenhouse seedlings and field plants were compared.

2.3.1. Fatty acid composition of tobacco seedlings. The oil of tobacco seeds and lipids of greenhouse seedlings were investigated regarding their fatty acid composition. Results in the range of C_{14} and C_{18} fatty acids are collected in Table 4.

Comparison of the oil of tobacco seeds and the fatty acid composition of the seedlings resulted in a discrepancy mainly in the ratio of linoleic ($C_{18:2}$) to linolenic ($C_{18:3}$) acid.

2.3.2. Initial phase of vegetative development. The values of fatty acid composition of the petroleum ether extract of field plants are given in Table 5.

In studying field plants, we measured the increased concentration of C_{16} palmitic acid and the appearance of unsaturated $C_{16:1}$ and $C_{16:2}$ fatty acids. In the 2nd week after plantation the ratio in C_{16} acids suddenly increased in the *Burley* variety, while the ratio in linolenic ($C_{18:3}$)acid decreased. Such a change can be observed in *Virginia* leaves only between the 4th and 6th weeks.

2.3.3. The phase of intensive development. The data obtained in the 6–12-week period are collected in Table 6.

With both varieties, there is a marked increase in the ratio of unsaturated fatty acids. In the case of oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids a certain increase could be observed, while the ratio of palmitic (C_{16}) acid considerably decreased during the same period. In the 12th week the ratio of total unsaturated C_{18} fatty acids reached 60% in variety *Virginia* and 70% in the *Burley*. We found significant differences in the distribution of saturated and unsaturated C_{14}–C_{18} fatty acids at various phases. Chromatograms of fatty acid methyl esters of variety *Virginia* from samples taken in the 8th and 12th week are presented in Figs. 7a and 7b resp.

In comparing the two chromatograms we may see a marked difference between the fatty acid composition of the intensive phase (8th week) and the final phase (12th week).

Table 4

The distribution of C_{14}–C_{18} fatty acids in the petroleum

	Sample	C_{14}		C_{16}		$C_{16:1}$	
		$\bar{X}$	s	$\bar{X}$	s	$\bar{X}$	s
Virginia	Seed	–	–	9.0	1.30	–	–
	Seedling 0 week	1.7	0.72	21.1	2.53	2.4	0.6
	Seedling 2nd week	1.6	0.60	17.2	1.25	2.6	0.7
Burley	Seed	–	–	9.4	1.47	–	–
	Seedling 0 week	3.4	0.87	15.8	1.25	3.2	1.1
	Seedling 2nd week	0.8	0.44	12.9	1.05	2.5	0.9

Results obtained on 10% DEGS stationary phase; evaluation of chromatogram $C_{16:1}$ palmitoleic acid; X = peak proportion on the chromatogram, values

Table 5

Changes in the distribution of C_{14}–C_{18} fatty acids of tobacco

	Sample	C_{14}		C_{16}		$C_{16:1}$	
		$\bar{X}$	s	$\bar{X}$	s	$\bar{X}$	s
Virginia	2nd week	1.6	0.72	15.8	1.30	2.2	0.70
	4th week	3.2	0.87	21.5	1.31	3.8	0.87
	6th week	5.2	0.82	41.7	2.17	2.4	0.78
Burley	2nd week	5.3	1.05	35.9	2.00	2.9	0.82
	4th week	7.3	1.11	31.0	1.65	7.3	1.05
	6th week	7.4	1.14	45.4	2.60	10.8	1.11

Results obtained on 10% DEGS stationary phase; evaluation of chromatogram chromatogram, values obtained from 3 parallel measurements;

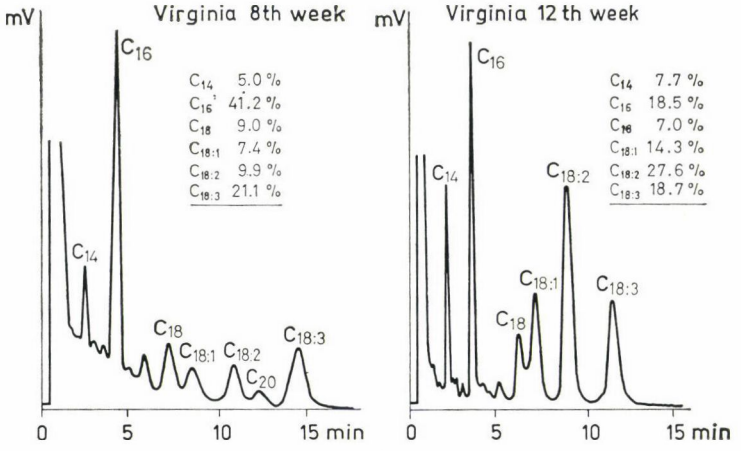

Fig. 7. Gas chromatogram of the fatty acid fraction of *Virginia* tobacco after the 8th and 12th weeks. Fatty acids were eluted in the form of methyl esters on 10% DEGS stationary phase at 185 °C, FID on *Chrom*-31 instrument

ether extract of tobacco seeds and seedlings

C_{18}		$C_{18:1}$		$C_{18:2}$		$C_{18:3}$	
$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s
3.2	0.82	9.9	1.21	75.8	2.88	2.1	0.82
4.3	1.05	8.4	1.11	17.8	1.21	42.3	2.69
3.1	0.87	7.8	0.78	15.8	1.30	51.8	3.02
3.2	1.21	12.8	1.47	73.5	2.92	1.0	0.72
2.9	1.05	10.5	1.11	13.2	1.13	52.9	2.52
5.0	0.82	17.0	1.14	14.8	1.01	47.0	2.60

according to the % of peak areas × (peak height half width); C_{14} = myristic acid; obtained from 3 parallel measurements; s = standard deviation (related to $\overline{X}$)

leaves in the initial phase of development (2–6 weeks)

$C_{16:2}$		C_{18}		$C_{18:1}$		$C_{18:2}$		$C_{18:3}$	
X	s	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s
3.0	0.60	1.9	0.72	7.2	1.39	14.3	1.65	54.2	2.92
2.5	0.70	3.7	0.82	4.9	1.05	13.6	1.51	49.3	2.47
3.1	0.79	6.5	0.87	8.5	1.56	10.1	1.31	21.0	2.09
9.8	0.89	4.4	1.25	7.4	1.30	13.5	1.05	23.4	2.35
9.8	0.92	3.2	0.82	6.0	0.74	11.6	1.93	24.5	2.88
4.2	1.11	6.4	1.01	4.2	0.82	11.2	1.02	7.2	1.05

according to the % of peak areas (peak height × half width); $\overline{X}$ = peak proportion on the s = standard deviation (related to $\overline{X}$)

2.3.4. Fatty acid composition in the final phase of development in bottom and top leaves. Data of fatty acid composition determined in the 12–16th week are summarized in Table 7.

The changes in the fatty acid composition of petroleum ether extracts of the lower, senescent leaves are not so marked as the qualitative changes observed during the phase of intensive development. Comparing the fatty acid composition of the upper and lower leaves, we may state that the ratio of C_{16} fatty acids is higher while that of unsaturated C_{18} fatty acids is lower. A study of the fatty acid composition of *Virginia* and *Burley* samples revealed that with the C_{18} fatty acids this difference is due to $C_{18:1}$ oleic acid and $C_{18:2}$ linoleic acid in variety *Virginia*, while the difference in the ratio of variety *Burley* may be attributed to the $C_{18:3}$ linolenic acid in the lower and upper leaves.

Table 6

Changes in the distribution of C_{14}–C_{18} fatty acids of tobacco

	Sample	C_{14}		C_{16}		$C_{16:1}$	
		$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s
Virginia	6th week	5.2	0.82	41.7	2.17	2.4	0.78
	8th week	5.0	0.30	41.2	2.77	1.5	0.72
	10th week	8.8	0.92	31.0	2.02	2.5	0.70
	12th week	7.7	0.89	18.5	1.31	1.7	0.62
Burley	6th week	7.4	1.14	45.4	2.60	10.8	1.11
	8th week	5.9	1.05	29.0	1.93	2.6	0.82
	10th week	4.5	0.82	19.8	1.57	2.1	0.70
	12th week	3.4	0.87	17.6	1.13	1.3	0.72

Results obtained on 10% DEGS stationary phase; evaluation of chromatogram chromatogram, values obtained from 3 parallel measurements;

Table 7

Changes in the distribution of C_{14}–C_1f fatty acids of tobacco leaves

	Sample	C_{14}		C_{16}		$C_{16:1}$	
		$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s
Virginia	12th week lower	7.7	0.89	18.5	1.31	1.7	0.62
	upper	5.2	1.07	27.2	1.54	5.2	0.89
	14th week lower	7.0	0.92	20.1	2.07	1.5	0.30
	upper	5.3	0.82	28.2	2.09	4.2	1.14
	16th week lower	6.5	0.87	21.8	1.82	1.4	0.44
	upper	5.5	0.89	27.0	1.76	3.3	0.62
Burley	12th week lower	3.4	0.87	17.6	1.13	1.3	0.72
	upper	6.2	1.05	25.0	2.35	4.2	0.82
	14th week lower	3.1	0.61	20.1	1.56	1.4	0.87
	upper	6.7	1.01	26.6	1.93	3.1	0.87
	16th week lower	2.3	0.62	23.0	1.51	1.4	0.60
	upper	7.5	0.87	28.2	2.00	1.1	0.60

$\overline{X}$ = peak proportion on the chromatogram, values obtained from 3 parallel

leaves in the intensive phase of development (6–12 weeks)

$C_{16:2}$		C_{18}		$C_{18:1}$		$C_{18:2}$		$C_{18:3}$	
$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s
3.1	0.79	6.5	0.87	8.5	1.56	10.1	1.21	21.0	2.09
4.7	1.07	9.0	1.56	7.4	1.31	9.9	1.02	21.1	2.07
3.7	1.13	6.4	1.21	11.7	1.30	14.5	1.74	21.4	1.82
2.2	0.70	7.0	1.02	14.3	1.47	18.7	1.76	21.9	2.35
4.2	1.11	6.4	1.01	4.2	0.82	11.2	1.03	7.2	1.05
1.6	0.72	5.3	0.82	12.5	1.05	10.8	1.50	31.8	2.07
0.6	0.56	4.0	0.87	10.2	1.56	11.3	1.39	45.5	2.80
1.5	0.61	5.9	1.15	10.2	1.74	17.1	2.53	43.0	2.81

according to the % of peak areas (peak height × half width); $\overline{X}$ = peak proportion on the
s = standard deviation (related to $\overline{X}$)

in the final phase of development (12–16 weeks) in lower and upper leaves

$C_{16:2}$		C_{18}		$C_{18:1}$		$C_{18:2}$		$C_{18:3}$	
$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s
2.2	0.70	7.0	1.02	14.3	1.47	18.7	1.76	21.9	2.35
2.4	0.87	8.8	1.11	12.1	1.30	14.9	1.82	24.2	2.07
2.0	0.70	6.5	1.05	14.0	1.21	22.1	1.74	26.9	2.09
2.0	0.78	9.3	1.12	10.0	1.11	14.1	1.56	26.9	1.82
1.1	0.60	5.9	0.87	13.5	1.56	18.9	1.47	30.9	2.52
2.2	0.70	10.4	1.01	9.5	1.31	13.5	1.25	28.6	2.69
1.5	0.61	5.9	1.15	10.2	1.74	17.1	2.53	43.0	2.81
1.8	0.70	8.8	1.05	15.4	1.93	15.1	1.56	23. 5	1.74
0.8	0.62	5.4	1.01	10.4	1.11	17.4	1.14	41.4	2.00
1.0	0.60	9.2	1.74	14.3	1.50	14.4	1.65	24.7	1.65
0.4	0.44	4.2	0.87	14.3	1.56	12.2	1.11	42.2	2.35
0.8	0.30	9.7	1.14	13.6	1.30	12.4	1.74	26.7	2.09

measurements; s = standard deviation (related to $\overline{X}$)

2.4. Study of C_{20}–C_{30} wax acids of the petroleum ether extract in the course of development

Gas chromatographic measurements of the longer-chain components of the fatty acid fraction could not be carried out reliably by the isothermic method described in the previous section, therefore we obtained data for components with longer-chain length by the temperature programming method using a less selective, but heat-stable stationary phase. At this apolar silicon oil phase no separation could be attained according to the number of unsaturated bonds only according to the number of carbon atoms. Chromatograms of so-called wax acids with higher carbon atom numbers taken in the 0th and 5th week are presented in Fig. 8.

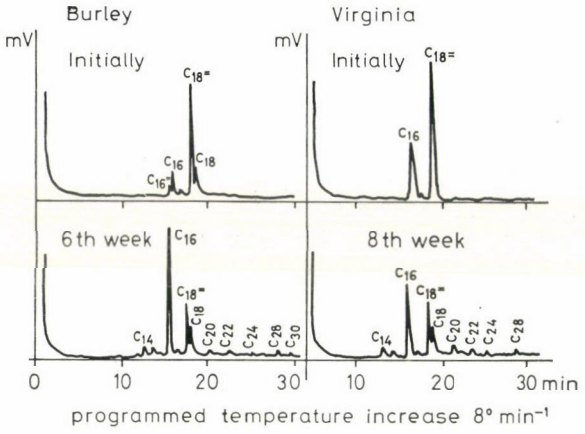

Fig. 8. Gas chromatogram of fatty and wax acid methyl esters from *Virginia* and *Burley* seedlings and 6–8 weeks old field plants. Stationary phase: 1% OV–101, temperature program 8 °C min^{-1}, in the range of 110–315 °C, FID on *Pye* 104 instrument

By comparing these chromatograms we could state that in the leaves of seedlings no C_{20}–C_{30} components could be measured while at the onset of the intensive development period a large number of wax acids (although not more than 10–15%) could be detected in both tobacco varieties. The change in the ratio of the total wax acid fraction plotted against time is given in Fig. 9.

The ratio of wax acids with a straight chain containing mainly C_{22}, C_{24}, C_{26}, C_{28} and C_{30} components in all fatty acids gives a higher maximum in variety *Virginia* compared to *Burley* at an earlier stage.

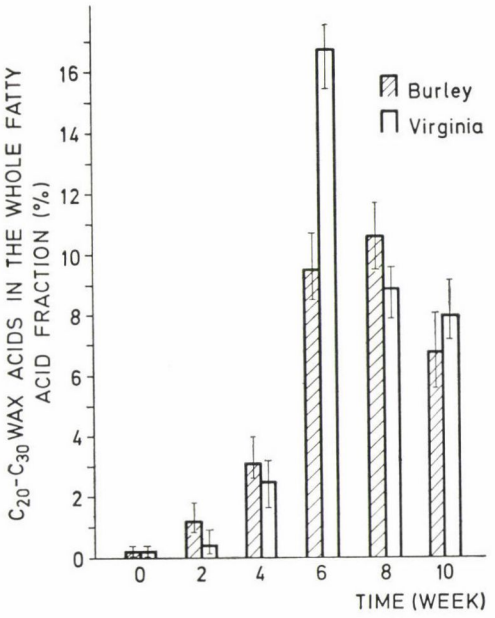

Fig. 9. Changes in ratio of wax acids (C_{20}–C_{30}) in the fatty acid fraction of the petroleum ether extract, summarized data from total peak area evaluation. Plotted values are the averages of 3 measurements ($n = 3$), extreme values shown by vertical lines

2.5. Study of lipid extracts with thin-layer chromatography

In addition to the detailed study of the fatty acid fraction, other qualitative changes were followed in both lipid fractions by thin-layer chromatography. Of the secondary substances in the neutral lipid fraction (petroleum ether extract) we studied in the first place sterols that play an important role in the mobilization of fatty acids. By running samples taken at different phenophases on the same plate, we found that the amount of free sterols and those esterified with fatty acids gradually decreases after planting out. In the phase of intensive development considerable amounts of the sterols are present in the form of fatty esters.

Much greater difference was observed in the thin-layer chromatographic study of the chloroform–methanol extract. This lipid extract contains lipids more strongly bound to leaf tissue. We estimated that 2/3 of the substances are polar lipids (phospholipids, glycolipids), whereas 1/3 are neutral lipids (triglycerides, hydrocarbons) and pigments.

After separating the polar lipids of seedlings and field plants, we identified phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, sterolglycosides and glycolipids on the plate, as illustrated in Figs. 10a and b. The proportion of sterolglycosides has suddenly decreased after planting,

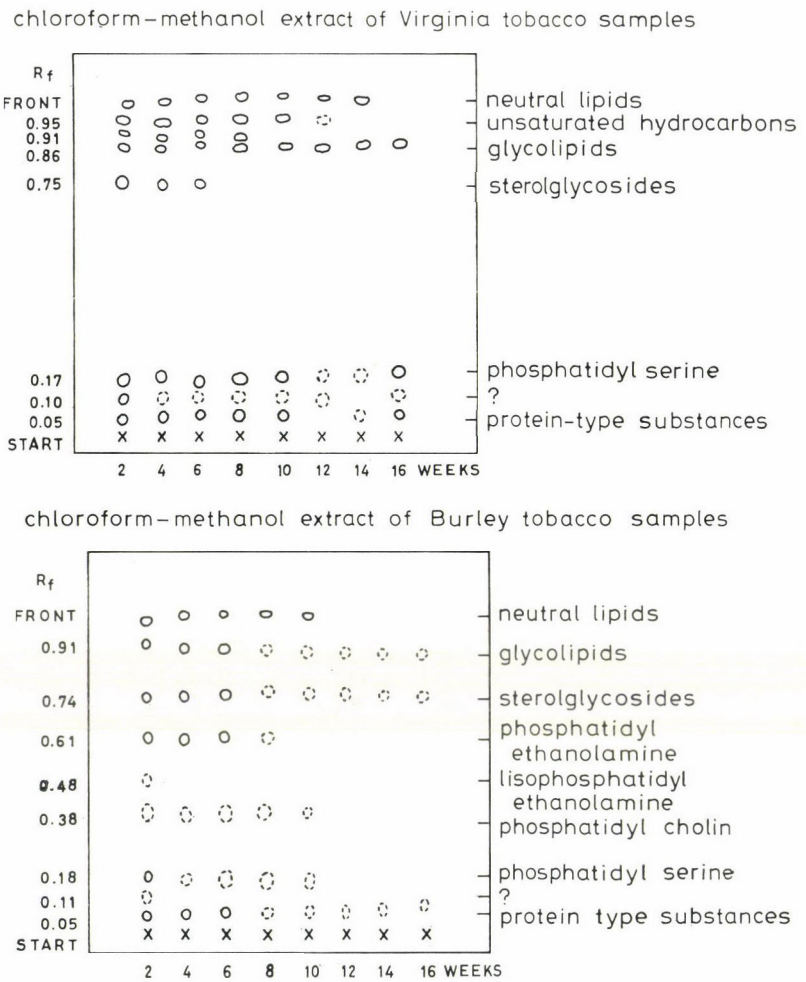

Fig. 10. Thin-layer chromatogram of the chloroform–methanol extract of *Virginia* (10a.) and *Burley* (10b.) tobacco samples obtained in the 2nd to 16th weeks. Lipid components were detected by spraying each plate with specific reagents (see text). Conditions: Kieselgel G, thickness 0.2 mm, layered from a solution of 0.01 *M* NaCl to 20 × 20 cm plates. Eluent: chloroform–methanol–water (65:25:4), 25 °C

while the level of polar lipids with phosphatidyl serine and glycolipids has not changed. Comparison of the *Burley* and *Virginia* varieties showed the former to be richer in polar lipids with mainly higher phosphatide content, while in the variety *Virginia* glycolipids gave more intensive spots on the plate. During the 12th and 16th week a relative increase in the phosphatidyl serine and glycolipids could be observed in the senescent leaves. The extract of upper leaves showed the presence of a higher amount of phosphatidyl serine and other

components with NH_2 content than found in lower leaves. In the 16th week, however, the amount of these components has also decreased. Detailed analysis of the chromatograms was made by comparing the spot sizes and colour intensities obtained by specific colour reactions on the same plate.

3. Conclusions

The diagrams showing vegetative development verify the observation of tobacco growers, according to which the growth of the *Virginia* variety is a steady slow process, while variety *Burley* first grows strong roots and then suddenly surpasses the *Virginia* plant. Comparison of our own data with results of other investigators revealed that there is a numerically evaluable difference in the rate of vegetative development of the two varieties and that the different phases of development can be well defined. It may be stated, therefore, that the whole process can be graphically divided into well-defined phases. In the course of our work, we attempted to find some correlation between these characteristics and the results of our investigations in lipid chemistry.

The planting of tobacco seedlings into fields resulted in a decrease in the amount of lipid extracts for both varieties. After the first period of adaptation to new field conditions, the amount of the petroleum ether extract gradually increased in the *Virginia* plant and showed a later, but rapid increase in variety *Burley*. In the phase of intensive development the amount of lipids, especially that of the chloroform–methanol extract, increased in both varieties. Lipid content reaches its maximum value at the final stage of vegetative development. This value is considerably higher for the *Virginia* plant than for variety *Burley* and is characteristic of technological ripeness, *i.e.* of the state of senescence. The presumption that lipids are formed in larger amount in the leaves of the slowly developing variety *Virginia*, has been supported by our experimental results. The lipid content of upper leaves reaches a maximum two weeks later, what is in agreement with the practice of breaking tobacco leaves.

The change in the ratio of the fatty acid part to the unsaponifiable fraction separated from the petroleum ether extract by saponification shows identical characteristics with both varieties. The ratio of fatty acid fraction is identical, approx. 50% in both plants and shows a steady decrease in the initial phase of vegetative development. It reaches minimum value in the 6th week with the *Virginia* and in the 8th week in the Burley plant, which is in agreement with the different stages of development plotted in the curve of vegetative growth. After a temporary increase following the final stage of development, in the course of senescence, a marked decrease could be observed in the ratio of the fatty acid fraction. This ratio is always higher in the upper leaves than in the lower ones. In comparing the two species, we may state that the accumulation

of fatty acids started earlier in the *Virginia* plant and thus, the ratio was never lower than 15% as was observed on the other hand for variety *Burley*. In the final phase, the ratio of fatty acid fraction was a higher value than that observed in *Burley* tobacco, however in the phase of senescence it decreased to the same value in both varieties.

Qualitative investigation of the fatty acid fraction revealed significant differences in each phenophase. In the petroleum ether extract of field plants, gas chromatographic investigations showed an accumulation in C_{16} saturated and unsaturated fatty acids, which points to the formation of polar lipids. The ratio of C_{18} fatty acid components containing unsaturated bonds showed a marked decrease in the initial phase of vegetative development and the synthesis of these components started only at the beginning of intensive development. Considerable increase can be observed especially in the ratio of oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids while the ratio of linolenic ($C_{18:3}$) acid and palmitic (C_{16}) acid showed a significant decrease in the final phase of vegetative development. It should be noted that the main fatty acid component for glycolipids is linolenic acid and for phospholipids it is palmitic acid. The change in the amount of these fatty acids shows that these components play a less significant role in the final phase of the process. Concentration of oleic and linoleic acids during the same period increases, on the other hand, and suggests the accumulation of triglycerides in the leaves in this period, and may serve as an indication for technological ripeness.

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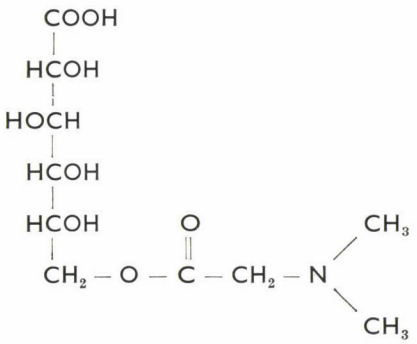
ERRATUM

RECENT DATA ON THE STRUCTURE OF PANGAMIC ACID (VITAMIN B_{15})

L. Telegdy Kováts, É. Berndorfer-Kraszner, Á. Juhász and T. Gábor

Volume 5, No. 3, p. 282, Fig. 1:



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